ROLE OF ETHYLENE IN LETTUCE (Lactuca sativa L.) GERMINATION AT SUPRAOPTIMAL TEMPERATURES

By

IWANKA KOZAREWA

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This work is dedicated to the memory of my father, Jordan Kozarew

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Iwanka Kozarewa

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Chair: Dr. Daniel J. Cantliffe
Cochair: Dr. Russell T. Nagata
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Lettuce germination is strongly dependent on the temperature of imbibition and subject to complex hormonal and developmental regulation. The objective of this research was to establish the role of ethylene in germination of thermosensitive 'Dark Green Boston' and thermotolerant 'Everglades' seeds at supraoptimal temperatures, in continuous light and dark. In order to achieve the research objective, the ethylene action inhibitor silver thiosulfate and the ethylene precursor , 1-aminocyclopropane-1-carboxylic acid, were applied to 'Dark Green Boston' and 'Everglades' seeds, and germination of transgenic lettuce lines with reduced ethylene perception or altered ethylene biosynthesis as well as seeds matured at elevated temperatures was examined. Germination of 'Dark Green Boston' seeds was stronger inhibited by silver thiosulfate than 'Everglades' germination. Silver thiosulfate was more effective in reducing germination at supraoptimal temperatures than at optimal temperatures.

Maturation of 'Dark Green Boston' and Everglades' seeds at elevated temperatures (30°/20°C) led to increased ethylene production, and consequently, germination as compared to seeds matured at 20°/10°C. Imbibition in dark led to reduced ethylene production as compared to light. Application of 1-aminocyclopropane-1-carboxylic acid increased germination of seeds matured at 20°/10°C in light to the level of 30°/20°C germination. 'Dark Green Boston' seeds with reduced ethylene perception were more thermosensitive than wild-type 'Dark Green Boston 'seeds. 'Everglades' seeds with reduced ethylene perception had similar germination to wild-type 'Everglades' seeds at optimal temperature in light and dark and at supraoptimal temperature in light. At supraoptimal temperature, in dark, their germination was significantly reduced as compared to wild type 'Everglades' germination. The results of the research indicated that the ability of lettuce seeds to germinate at supraoptimal temperatures was dependent on the ethylene production and was reduced if sensitivity to ethylene was decreased.

CHAPTER 1
INTRODUCTION

Lettuce (*Lactuca sativa* L.) is an important salad crop that is grown year round in US from either direct seeding or transplants. In both cases, lettuce seeds are often exposed to higher than the optimal temperatures, which results in poor and nonuniform germination. The optimum temperature for germination of many lettuce cultivars is between 15°C and 22°C (Cantliffe et al., 2000). If lettuce seeds are exposed to supraoptimal temperature, two physiological phenomena occur. Initially, lettuce seeds can germinate if transferred from supraoptimal temperatures to optimal ones. However, if the seeds are imbibed at supraoptimal temperatures for more than several days, they will not germinate even if returned to optimal temperatures. The first phenomenon is termed thermoinhibition and the second one thermodormancy.

Lettuce seeds have been one of the most used model systems for studying germination. In an extensive study, Sung et al. (1998) established that the ability of lettuce seeds to germinate at high temperatures depended equally on heritable and environmental factors. The authors identified several lettuce genotypes, expressing stable thermotolerance (e.g. 'Everglades') and several consistently unable to germinate at supraoptimal temperatures (e.g. 'Dark Green Boston'). The same group also reported that the thermotolerant character of lettuce seeds could be completely suppressed or the thermotolerance could be enhanced in certain lettuce genotypes, including 'Everglades' and 'Dark Green Boston', depending on temperature during seed maturation. Sung

et al. 1998, also identified the endosperm as the tissue restricting most radicle protrusion in lettuce and observed endosperm weakening prior to germination.

The mechanism of lettuce germination and especially its control by plant hormones was further studied by Nascimento et al. (2000, 2001). Since ethylene has been long reported to be more effective that other hormones in overcoming lettuce thermoinhibition and thermodormancy, the focus of this group was on the role of ethylene in lettuce germination. Nascimento et al. (2004a) reported that application of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), increased germination of thermosensitive 'Dark Green Boston' seeds to the level of thermotolerant 'Everglades' seeds at supraoptimal temperature. The authors also observed that seeds from thermotolerant lettuce genotypes produced more ethylene during germination at both optimal and supraoptimal temperatures than thermosensitive lettuce seeds. Ethylene production was increased during light imbibition as compared to imbibition in dark (Saini et al., 1989; Nascimento et al., 2000). Because of the observed endosperm weakening prior to germination and the polysaccharide composition of lettuce endosperm (composed predominantly of mannan polymers), the activity of the cell wall weakening enzyme, endo-beta-mannanase, prior to germination was examined. Endo-beta-mannanase activity was higher in thermotolerant lettuce seeds than in thermosensitive ones and also was enhanced by treatments enhancing germination (Nascimento et al., 2000; 2001). Nascimento et al. (2000) also hypothesized that ethylene might participate in regulation of endo-beta-mannanase based on the correlation of ethylene production and endo-beta-mannanase activity prior to germination.

The objective of this research was to establish a more definite role for ethylene and how it may regulate germination of thermosensitive and thermotolerant lettuce genotypes at supraoptimal temperatures. In order to do so, several experimental approaches were taken. These were: 1) examine the effect of reduced ethylene perception, imposed by the ethylene action inhibitor, silver thiosulfate, on germination of thermosensitive and thermotolerant lettuce seeds at supraoptimal temperatures in light; 2) identify the effect of reduced ethylene perception or altered ethylene production in transgenic lines of thermosensitive and thermotolerant lettuce genotypes on their seed germination at supraoptimal temperatures in either light or dark; 3) examine the effect of reduced ethylene perception or altered ethylene production in the transgenic lines on ethylene production during germination under different experimental conditions; 4) establish the effect of seed maturation at elevated temperatures on ethylene production, ethylene perception, and germination of thermosensitive and thermotolerant lettuce genotypes at supraoptimal temperatures.

CHAPTER 2
REVIEW OF LITERATURE

Lettuce (*Lactuca sativa* L.) is an important vegetable crop. Its production in US in 2002 was 3,071,980 metric tons (USDA, National Agricultural Statistics Service). In the US, lettuce is produced year-round from either direct field seeding or transplants. In both cases, high soil temperatures can cause nonuniform germination and non-optimal stand establishment. A technique called priming has been used commercially to improve lettuce germination. Priming is defined as a pre-sowing treatment in which seeds are soaked in an osmotic solution that allows them to imbibe water and go through the first stages of germination, but does not permit radicle protrusion through the seed coat (Heydecker et al., 1973). However, primed lettuce seeds deteriorate faster in storage than non-primed seeds (Tarquis and Bradford., 1992; Nascimento and Cantliffe, 1998).

Lettuce Seed Structure

A mature lettuce seed (achene) contains the following parts (Figure 2-1; Cantliffe et al., 2000):

1. Pericarp, or fruit coat, located on the outer surface of the seed, consisting of nonliving, lignified cells.
2. Integument, also a nonliving structure, located beneath the pericarp and composed of remnants of outer epidermis and some parenchymatous cells.
3. Endosperm, a living tissue, comprising about 8% of lettuce seed dry weight.

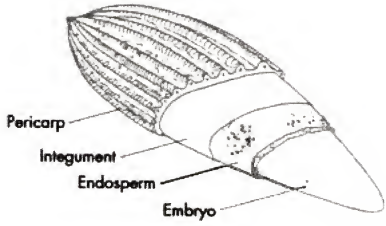


Figure 2-1. Diagram of a lettuce seed.

The endosperm cell wall contains approximately two-thirds of the total seed cell wall polysaccharide material (Halmer et al., 1975). The predominant noncellulosic sugars are galactomannans (Nascimento and Cantliffe, 2001).

Through experiments aimed at measuring the resistance of different seed parts to radicle growth (puncture tests), it was established that the endosperm was the barrier restricting lettuce germination. Thus, its structure and composition have been studied extensively (Georghiou et al., 1983, Psaras, 1984; Sung, 1996). Lettuce endosperm can be divided into two parts: a micropylar section, which is opposite the radicle tip, and a lateral part. The lateral portion consists of two distinct cell layers while the micropylar portion consists of three or more cell layers (Cantliffe et al., 2000). These two parts differ compositionally. Dutta et al. (1994) reported that the micropylar section of 'Pacific' lettuce seeds contained higher proportions of arabinose and glucose as compared to the lateral section. Nascimento and Cantliffe (2001) reported that mannose was the predominant noncellulosic sugar in both the micropylar and lateral regions of three lettuce genotypes, 'Dark Green Boston', 'Everglades', and 'PI 251245'. However, the micropylar endosperm of all three genotypes contained significantly higher amounts of rhamnose, arabinose, glucose and galactose as compared to the lateral region.

Factors Affecting Lettuce Germination

Germination is defined as a process that starts with water uptake by the seed and finishes with radicle protrusion (Bewley and Black, 1994). There are three phases of germination in which DNA and preexisting mitochondria are repaired, new mitochondria are synthesized, enzymes are re-activated or synthesized *de novo*, embryo growth is

initiated, and eventually seedling growth begins (Bewley, 1997). Lettuce germination is under control of both environmental (temperature and light) and genetic factors.

Temperature

Lettuce germination decreases with an increase in temperature from around 20°C to above 27°C. The optimum germination temperature for most lettuce cultivars is between 15ºC and 22ºC (Cantliffe et al., 2000). In many cultivars, temperatures above 25ºC lead to reduced germination and decreased germination speed. Above 27ºC germination of many lettuce cultivars is totally inhibited. When lettuce seeds are imbibed at supraoptimal temperature, two phenomena occur. During short exposure to supraoptimal temperature, the seeds will not germinate, but when returned to favorable temperature, they will germinate. This delay in germination is termed thermoinhibition. Seeds imbibed at supraoptimal temperature for several days will not germinate even when returned to optimal temperature. These seeds have entered a secondary dormancy, called thermodormancy.

Gray (1977) reported that the first four hours of seed imbibition were most sensitive to high temperature. Takeba and Matsubara (1976) found that when lettuce seeds were imbibed initially at low temperature, they were able to germinate when subsequently transferred to supraoptimal temperature. These results suggest that a thermo-labile factor controlling germination may be synthesized during the first hours of imbibition.

Restriction of germination by high temperature has been attributed to different factors, including a decrease in production or sensitivity to certain plant hormones,

inability to develop sufficient osmotic potential, or inhibition of synthesis of cell wall weakening enzymes. Abeles (1986) reported that 'Grand Rapids' lettuce seeds produced more ethylene at optimal temperature (25°C) than at supraoptimal temperature (30°C). Nascimento et al. (2000) found that imbibition at supraoptimal temperature reduced ethylene production in thermosensitive lettuce genotypes 'Dark Green Boston' and 'Valmaine' and increased ethylene production in the thermotolerant genotype 'Everglades'. The activity of the cell wall weakening enzyme, endo-beta-mannanase (EBM), was suggested to play an important role in lettuce germination. The levels of this enzyme were lower at non-optimal temperature. The disappearance of small fat bodies, a phenomenon associated with germination in lettuce, was also inhibited in thermodormant seeds (Psaras et al., 1981). Sung (1996) suggested that the disappearance of these bodies was involved in development of turgor pressure, necessary for radicle protrusion. Takeba (1980) reported that the amino acids glutamine and glutamate accumulated before radicle protrusion in 'New York' lettuce seeds and hypothesized that they might act as osmotic substances. This accumulation was much lower at supraoptimal temperature. Activity of glutamine synthetase also decreased rapidly during incubation at high temperature (Takeba,1983a).

Light

Germination of many lettuce cultivars (termed photosensitive) is strongly promoted by exposure to light (Bortwick and Robbins, 1928). Phytochrome is a protein that functions as a light receptor in plants. It exists in two forms: Pr, the red light-absorbing form, and Pfr, the far-red absorbing form. Pfr is the physiologically active

form. Lettuce seed germination is promoted even from a brief exposure to light. The promotive effect of the light is reversible. When 'Grand Rapids' seeds were exposed to a brief pulse of red light (2 min), germination increased from 10% in dark to 76% (Takeba, 1983b). However, if the red light pulse was followed by exposure to far-red light, the promotive effect of the red light was lost. Takeba (1983b) reported that the ability of lettuce seeds to germinate was dependent on the last light exposure. When a series of red and far-red irradiations was ended by red light irradiation, 75% of 'Grand Rapids' seeds germinated at 27°C. However, when the seeds were exposed last to far-red irradiation, their germination decreased to 8%. The same author reported an existence of an escape curve for the response of lettuce seeds to light, meaning that after a period of time following the last exposure, lettuce germination was no longer inhibited by far-red irradiation.

Light improves lettuce germination at supraoptimal temperature. Takeba (1984b) reported that germination of both 'New York 515 Improved 'and 'Grand Rapids' seeds at 30°C was above 80% in continuous red light compared to 0% in the dark. However, the ability of light to alleviate thermoinhibition and thermodormancy is limited and at temperatures equal to or higher than 35°C, light alone will not promote germination.

One of the ways through which light may promote germination is through stimulation of biosynthesis and/or sensitivity to gibberellins. Application of gibberellins can promote dark germination of seeds from many plants species, including lettuce, tomato and *Arabidopsis thaliana* L. Toyomasu et al. (1998) reported that red-light irradiation increased the endogenous content of GA_1, the bioactive gibberellin in lettuce. Since levels of its direct precursors, GA_{20} and GA_{19}, were not affected, this author

concluded that the conversion of GA_{20} to GA_1 was most likely regulated by light. In support of this, an induction of the expression of GA 3-beta-hydroxylase (an enzyme participating in the conversion of GA_{20} to GA_1) was reported after red-light treatment and was reversed in dark. In lettuce, no light effect on sensitivity to gibberellins was reported. Light-imbibed *Arabidopsis* seeds germinated at lower doses of GA_{4-7} than dark-imbibed seeds (Yamaguchi and Kamiya, 2001).

The promotive effect of light on germination might also be related to alleviation of the inhibitory effect of abscisic acid (ABA). Kraepiel et al. (1994) studied a phytochrome-deficient mutant (*pew1*) of *Nicotiana plumbaginifolia* L and found that the mutant plants contained higher amounts of ABA in seeds and leaves as compared to wild-type. Based on analysis of the mutant phenotype, they speculated that light might enhance ABA degradation.

The promotive effect of light on lettuce germination has been attributed to stimulation of ethylene production. Saini et al. (1989) reported that 'Grand Rapids' seeds imbibed under continuous red light at 32°C produced two to three times more ethylene than dark-imbibed seeds. Nascimento et al. (2000) also reported that 'Everglades' seeds produced more ethylene in light than in dark.

Genotype

The ability of lettuce seeds to germinate at supraoptimal temperature is genotype-dependent. Lettuce cultivars differ strongly in their upper limit for germination and can be divided into thermosensitive and thermotolerant. 'Dark Green Boston', a thermosensitive cultivar, does not germinate above 28°C while 'Everglades', a

thermotolerant cultivar, will germinate at 36°C. Gray (1975) and Thompson et al. (1979) tried to correlate the ability for germination at supraoptimal temperature with lettuce head type, preference for cultivation season, and seed coat color. However, no strong correlation was observed.

Thermotolerance is a heritable trait that can be introduced via breeding. In lettuce, this trait is controlled by at least two genes (Nagata, personal communication). In most of the commercial thermotolerant cultivars, thermotolerance was introduced from the Spanish cultivar 'Maturo' (Sung, 1996).

The basis of the genotype-dependent thermotolerance is not yet clear and is probably related to more than one factor. Prusinski and Khan (1990) reported that the ability of different lettuce cultivars to germinate under a variety of stress conditions (high temperature, salt and osmotic stress) depended on ethylene production. Heritable thermotolerance may also be related to factors other than hormonal balance. Srikanthbabu et al. (2002) observed that pea genotypes, capable of obtaining thermotolerance had higher expression of several heat shock proteins.

Environmental Conditions During Seed Production and Maturation

Temperature and light conditions during seed production and maturation affect the ability of lettuce seeds to germinate at supraoptimal temperatures. Koller (1962) first observed that seeds matured under high temperatures (30°/23°C) had better ability to germinate at supraoptimal temperature (26°C). Gray et al. (1988b) reported that seeds from the crisp head cultivar 'Saladin' matured at 30°/20°C day/night temperatures (D/N) germinated better at 30°C than seeds produced at either 25°/15°C or 20°/10°C. Sung

(1996) reported that germination of lettuce seeds from the thermotolerant genotypes 'Everglades' and 'Floricos 83' and the thermosensitive genotype 'Dark Green Boston' matured at 30°/20°C was better at supraoptimal temperatures (30°, 33° and 36°C) than that of seeds matured at either 25/°15°C or 20°/10°C. Also, 'Dark Green Boston' and 'Everglades' seeds matured at 30°/20°C germinated more rapidly than seeds produced at 25/°15°C and 20°/10°C at both optimal and supraoptimal temperatures. However, the same author reported that germination of the thermosensitive 'Valmaine' was not improved by higher maturation temperatures. This suggests that the ability of higher maturation temperatures to circumvent thermotolerance is genotype-dependent.

The ability of higher temperatures to improve germination may be related to an increase of DNA and RNA content and, consequently, enhancement of protein synthesis. Gray et al. (1988a) reported that carrot seeds matured at 30°/20°C had higher quantities of DNA and rRNA than seeds matured at 25°/15°C or 20°/10°C. The endosperm is considered to serve as a barrier during lettuce germination, especially at high temperature (Nascimento et al., 2000). Sung (1996) observed more structural alterations of the endosperm of both 'Dark Green Boston' and 'Everglades' seeds matured at 30°/20°C as compared to those matured at 20°/10°C. Seeds produced at 30°/20°C had cracks on one side of the endosperm and sometimes the endosperm layer was separated from the rest of the tissue. These seeds also had fewer cells in the micropylar endosperm as compared to seeds matured at 20°/10°C. Nascimento et al. (2000) hypothesized that the activity of EBM was necessary for endosperm weakening in lettuce seeds. These authors observed higher EBM activity immediately prior to or after germination in both 'Dark Green

Boston' and 'Everglades' seeds matured at 30/°20°C as compared to those matured at 20°/10°C.

The effects of different maturation conditions on seed dormancy and germination were also reported for other plant species. Sharif-Zadeh and Murdoch (2000) reported that dormancy of both caryopses and spikelets of *Cenchrus ciliaris* decreased with increased maturation temperature. Clapham et al. (2000) reported that maturation temperatures affected various parameters of seed vigor in white lupin, including germinability at different temperatures.

Hormonal Control of Lettuce Seed Germination

Gibberellins (GAs)

Gibberellins play an important role in lettuce germination under both optimal and inhibitory conditions. Germination is strongly reduced or completely inhibited in GA-deficient mutants. The extent of the germination inhibition corresponds to the severity of the GA-deficiency. In lettuce, the role of GAs has been most extensively studied in relation to their ability to release photodormancy (the inability of photosensitive lettuce seeds to germinate in dark). Application of GAs can completely circumvent the need for light. Takeba (1984b) reported that 'New York 515 Improved' seeds germinated at 40% at 25°C, in the dark, as compared to 100% in GA. Yamaguchi and Kamiya (2001) hypothesized that light promoted lettuce germination through up-regulation of GA biosynthesis. The same authors observed an increase in endogenous GA_1 (the major bioactive GA during lettuce germination) but not in its immediate precursor GA_{20} after red-light treatment. However, application of GA alone can not improve germination of

thermosensitive lettuce cultivars at supraoptimal temperature. Takeba (1984b) reported that 'New York 515 Improved' seeds did not germinate at 30°C in dark. However, these seeds germinated at 70% in dark and presence of GA, 80% in light and 90% in light and GA. At 35°C, the seeds did not germinate regardless of the treatment.

Gibberellins promote germination and post-germinative mobilization of stored reserves in many plant species. Both *Arabidopsis thaliana* and tomato GA-deficient mutants (*ga1* and *gib,* respectively) do not germinate unless supplied with exogenous GAs (Hilhorst and Karssen, 1992). Groot et al. (1988) reported that wild-type *Arabidopsis* and tomato seeds failed to germinate when imbibed in paclobutrazol or uniconazole, indicating that *de novo* GA synthesis was essential for germination. White et al. (2000) reported that GAs participated in establishing and maintaining primary dormancy in maize. These authors observed complete suppression of vivipary in double mutant (both ABA-insensitive and GA-deficient) maize kernels. The same group also reported that application of paclobutrazole suppressed vivipary in ABA-insensitive maize seeds. Application of GAs circumvented the need for treatments breaking the primary dormancy such as exposure to light, chilling and dry after-ripening in many plant species. Hilhorst and Karssen (1992) hypothesized that chilling and dry after-ripening might promote germination through increasing the sensitivity to endogenous GAs. GA also participates in the release from seed coat or endosperm imposed dormancy. Hilhorst and Karssen (1992) reported that removal of these seed parts allowed gibberellin-deficient tomato seeds to germinate. Also, application of exogenous GAs lowered the mechanical resistance of the testa. Debeaujon and Koornneef (2000) reported that *Arabidopsis thaliana* seeds produced from crossing mutant seeds with weakened testa (*tt* line) to GA-

deficient seeds (*ga1*) were able to germinate without need for exogenous GA. However, GAs have not been reported to promote germination specifically under inhibitory conditions.

GA promotes germination through several different mechanisms. Karssen and Lacka (1986) hypothesized that GAs regulated the processes leading to development of osmotic potential. In lettuce, Takeba (1980) hypothesized that an increase in growth potential was related to accumulation of free amino acids (especially glutamine and glutamate). Growth potential is determined as the osmotic potential required to achieve 50% germination (Scheibe and Lang, 1965). Takeba (1980) also identified glutamine synthetase as a key enzyme in this process. He reported that application of 1 mM GA to 'New York' lettuce seeds in dark at 27°C increased glutamine synthetase activity from 0.92 to 1.42 and, at the same time, increased germination from 8% to 92%.

In seeds with coat-imposed restrictions of germination, GAs may promote the germination through enhancement of the activity of cell wall weakening enzymes. A system in which the promotive effect of GAs on endosperm weakening has been extensively studied is the tomato seed. In 1987, Groot and Karssen, using gibberellin-deficient tomato seeds (*gib*1), observed regulation of endosperm weakening by GAs. The authors hypothesized that GAs were excreted from the embryo to the micropylar endosperm where they reduced the mechanical resistance of the endosperm. Groot et al. (1988) observed induction of EBM activity in isolated tomato endosperm following GA_{4+7} application. Based on this observation, the authors hypothesized that endosperm weakening in tomato is mediated by EBM. Nonogaki et al. (2000) reported that the EBM activity was absent in *gib1* seeds imbibed in water and detectable after application of

exogenous GA. Watkins et al. (1985) reported higher activity of galactomannan degradating enzyme(s) and higher germination in pepper seeds imbibed in GA as compared to those imbibed in water.

GAs were also reported to promote germination through maintaining germination-favorable conditions. Cooley et al. (1999) reported that GAs stimulate the activity of vacuolar H^+-ATPase and thus participate in decreasing the cytosolic pH to a range optimal for the cell wall weakening enzymes.

Abscisic Acid (ABA)

ABA participates in regulation of both dormancy and germination of lettuce seeds. The ability of lettuce seeds to overcome primary dormancy and to germinate has been correlated with levels of endogenous ABA. Toyomasu et al. (1994) reported that germination promoting treatments (red-light irradiation) and GA application decreased ABA levels. ABA participates in regulation of lettuce germination under inhibitory conditions. Yoshioka et al. (1998) hypothesized that maintenance of high ABA content was responsible for high-temperature inhibition of lettuce germination. These authors reported that endogenous ABA levels decreased in lettuce seeds after imbibition at 23°C but did not do so at 33°C. Application of fluoridone, an ABA biosynthesis inhibitor, restored seed germination at supraoptimal temperature. However, Khan (1994a) reported that application of ABA alone failed to impose dormancy in lettuce at different germination conditions (25°C in dark, 5°C in dark and osmotic stress). He concluded that more factors than ABA participated in dormancy induction and maintenance in lettuce.

ABA plays an important role in seed development and germination in other plant species. Mutant *Arabidopsis thaliana*, tomato, and maize seeds, which are unable to produce ABA or respond to ABA, do not enter primary dormancy or even germinate while still attached to the mother plant. Grappin et al. (2000) reported that *Nicotiana plumbaginifolia* L. ABA-deficient seeds did not require a period of after-ripening to germinate as did their wild-type counterparts. Beaudoin et al. (2000) reported that *Arabidopsis* seeds, hypersensitive to ABA, had deeper dormancy than wild-type seeds. However, increased production of ABA or maintenance of high levels of ABA did not always coincide with inhibition of germination. Ni and Bradford (1992) reported that inhibition of tomato germination by low water potential was not related to an increase in endogenous ABA content.

It is hypothesized that ABA inhibits seed germination through several different mechanisms. In many plant species (lettuce, tomato, pepper, and tobacco), germination is restricted by the tissues surrounding the radicle. The activity of some cell wall weakening enzymes is inhibited by ABA. Dulson et al. (1988) reported that ABA inhibited mannanase production in isolated lettuce 'Grand Rapids" endosperms. This group negatively correlated the endogenous ABA content with mannanase production under different experimental conditions. Toorop et al. (2000) mentioned that ABA inhibited the activity of beta-1, 3-glucanase, which participated in tobacco germination. The ability of ABA to inhibit germination through inhibition of activity of cell wall weakening enzymes has been extensively studied in tomato. Groot and Karssen (1992) reported an inhibitory effect of ABA on endosperm weakening in tomato seeds. Nomaguchi et al. (1995) observed inhibition of mannanase activity in the presence of ABA. Although other

authors observed inhibition of tomato endosperm weakening when ABA was present, they did not observe an accompanying inhibition of endo-beta-mannanase activity (Toorop et al., 1996). Toorop et al. (2000) reported that endosperm weakening was observed in tomato seeds imbibed in both water and ABA until the 45th hour of imbibition. Afterwards, the endosperm of seeds imbibed in water continued to weaken while the weakening of endosperms in ABA ceased. However, endo-beta-mannanase activity continued to increase even after endosperm weakening stopped under prolonged ABA treatment. Transfer of seeds from ABA to water did not result in increased endo-beta-mannanase activity. This clearly indicates that the ABA-mediated inhibition of endosperm weakening is not related to endo-beta-mannanase. Furthermore, Toorop (1998) reported that none of the enzymes with hypothesized importance for tomato endosperm weakening (α-galactosidase, β-mannosidase, cellulose, β-glucosidase or exo-polygalacturonase) were inhibited by ABA. The mechanism of ABA-mediated inhibition of tomato endosperm weakening remains unknown.

The second mechanism through which ABA may inhibit germination is by decreasing the seed growth potential. Ni and Bradford (1993) reported that tomato ABA-deficient seeds germinated more quickly and uniformly in water than wild-type seeds. Schopfer and Plachy (1985) reported that ABA might maintain dormancy through specifically preventing the embryo from entering the growth phase of germination. Cooley et al. (1999) reported that ABA down-regulated expression of vacuolar H^+-ATPase, which might lead to the creation of unfavorable germination conditions.

Germinating seeds have developed different mechanisms to counteract the effect of ABA. The effect of germination promoting treatments has often been attributed to

decreasing ABA biosynthesis or increasing ABA degradation. Grappin et al. (2000) and Le Page-Degivry et al. (1992) reported that application of fluridone induced germination of dormant seeds of *Nicotiana plumbaginifolia* L. and sunflower, respectively. Le-Page-Degivry et al. (1997) reported that cold pretreatment of dormant embryos of *Fagus sylvatica* modified the equilibrium between ABA synthesis and degradation by increasing the rate of ABA degradation. Barthe et al. (2000) reported that ABA catabolism was strongly oxygen dependent and non-metabolized ABA accumulated in the absence of oxygen. This group hypothesized that one of the mechanisms of testa-imposed dormancy might be restriction of oxygen intake and, consequently, ABA degradation. Maintenance or release from dormancy is also related to differences in sensitivity to ABA. Juricic et al. (2000) reported that immature rape seeds had high sensitivity to exogenous ABA and that this sensitivity decreased upon maturation. Schmitz et al. (2000) reported that dormancy-breaking treatments decreased sensitivity of yellow-cedar seeds to increasing ABA concentrations. Sensitivity was decreased proportionally to the treatment duration.

ABA interacts with other hormones and other environmental factors during its action. ABA and GA have been reported to have antagonistic effects on seed maturation and germination. White et al. (2000) reported that the relative amount of GA and ABA rather that their concentrations alone determined whether developing maize embryos would undergo precocious germination or maturation. Application of GA biosynthesis inhibitors early in embryogenesis suppressed vivipary in ABA-deficient maize kernels. Vivipary was also suppressed in ABA-insensitive and GA-deficient double mutant kernels. Similar to the antagonistic interaction between GA and ABA is the interaction between ABA and light. Roth-Bejerano et al. (1999) reported that ABA-mediated

inhibition of lettuce germination was circumvented by brief exposure to light. Also, germination of the light-sensitive lettuce cultivar Ritsa was inhibited by lower concentrations of ABA than that of the light-insensitive cultivar Strada.

Cytokinins

Cytokinins have a promotive effect on germination of many plant species, under both optimal and inhibitory conditions. The effect of exogenously applied kinetin on germination has been studied in lettuce seeds. Kinetin improved germination of 'Mesa 659' seeds at optimal conditions (25°C in light) by increasing the germination rate (Khan and Prusinski, 1988; Khan and Huang, 1989). At inhibitory conditions (supraoptimal temperature or presence of 0.1 M NaCl) kinetin increased the rate of germination and total germination. Khan and Prusinski (1988) reported that with an increase in the severity of the stress, the promotive effect of kinetin decreased. Lettuce seeds soaked in 0.05 mM kinetin germinated at 93% at 25°C as compared to 75% at 32°C and 8% at 35°C. Khan and Prusinski (1988) reported that exogenous cytokinins broke dormancy of peanut seeds. Rinaldi (2000) reported that application of thidiazuron, a compound with cytokinin-like activity, stimulated germination of seeds from six different olive cultivars.

The promotive effect of cytokinins on germination has been attributed to two different processes. Khan and Huang (1988) reported that in lettuce seeds exogenous cytokinins increased ethylene production. These authors hypothesized that cytokinins stimulated both production and utilization of 1-aminocyclopropane-1-carboxylic acid (ACC). Herrera-Teigeiro et al. (1999) proposed a different mechanism of promoting

germination. These authors observed stimulation of cell division following application of cytokinins.

Cytokinins interact with other hormones or environmental factors during germination. Thomas et al. (1997) reported that cytokinins were able to break dormancy in light-requiring lettuce seeds only if these seeds were exposed briefly to red light or if GA was present. The same group reported that application of light increased endogenous cytokinin levels in seeds of *Apium graveolens, Picea sitchensis, Pinus sylvestris,* and *Rumex obtusifolius*. Gusta et al. (1982) reported that cytokinins were able to counteract ABA and maintained ethylene production in water-stressed wheat leaves and ABA-treated plant tissues.

Role of Ethylene in Lettuce Germination

Ethylene is one of only a few gaseous plant hormones. It participates in regulation of many plant physiological processes, including flowering, fruit ripening, senescence, and abscission. The role of ethylene in germination has been studied extensively in several plant species, including lettuce.

Ethylene and Lettuce Germination

The importance of ethylene for lettuce germination at optimal temperature is still controversial. It has been suggested that ethylene is necessary for germination at optimal temperature but that the requirements for ethylene are very low and cultivar-dependent (Nascimento et al., 2000). Abeles (1986) reported that application of an ethylene production inhibitor, aminoethoxyvinylglycine (AVG) at 1 mM concentration, reduced

by 50% both ethylene production and germination of 'Grand Rapids' lettuce seeds at 25°C. However, Nascimento et al. (2004a) reported that application of 10 mM AVG inhibited ethylene production in 'Everglades' and 'Dark Green Boston' seeds but did not affect germination at 20°C in light. Studies using ethylene action inhibitors have also resulted in controversial results. Abeles (1986) reported that the ethylene action inhibitor 2, 5-norbornadiene (NBD) was effective in inhibiting germination of 'Grand Rapids' seeds at 25°C. However, application of another ethylene action inhibitor, silver thiosulfate (STS) at a concentration of 1 mM, did not reduce germination at 25°C. Nascimento et al. (2004a) described noninhibited germination of thermotolerant 'Everglades' seeds at 20°C by a 20 mM STS. However, germination of the thermosensitive genotype 'Dark Green Boston' was reduced from 98% in water to 77% in STS. The discrepancy in the effect of the ethylene inhibitors might be explained by a difference in the amount of ethylene produced by the different lettuce cultivars. Nascimento et al. (2000) reported that 'Everglades' seeds produced significantly more ethylene than 'Dark Green Boston' seeds at 20°C.

Ethylene plays a significant role in lettuce germination under stressful conditions (high temperature, salt stress, and presence of osmoticum). Abeles (1986) reported that inhibitors of both ethylene production (AVG) and ethylene action (NBD, STS) reduced 'Grand Rapids' germination at 30°C. Saini et al. (1989) reported that application of 1 mM AVG partially inhibited germination while application of 2 ml/L NBD completely inhibited germination of 'Grand Rapids' seeds at 32°C in continuous red light. The same authors observed that a combination of 1 mM AVG and 1 ml/L NBD was as effective in preventing germination as 2 ml/L NBD. Saini et al. (1989) hypothesized that the

requirements for ethylene at 32°C might be low and satisfied by residual ethylene production in the presence of AVG. Only if any remaining ethylene was prevented from action by NBD, then the germination was completely inhibited. Nascimento et al. (2004a) reported that application of 20 mM STS reduced germination of both 'Dark Green Boston' (from 33% to 0%) and 'Everglades' (from 98% to 49%) cultivars at 35°C in light. The same author also observed a significant increase, from 33% to 92%, in germination of 'Dark Green Boston' seeds at 35°C in light when they were imbibed in 10 mM ACC. Prusinski and Khan (1990) reported that ethylene production by slit seeds from nine lettuce cultivars correlated well with their ability to germinate at both 32°C and 35°C. In the same study, a strong correlation between ethylene production and germination under other types of stress (osmotic and salt stress) was reported. Zapata et al. (2003) also reported that seeds from lettuce cultivars with low tolerance to 150 mM NaCl had low ethylene production.

With age, the ability of lettuce seeds to germinate under stressful conditions decreases, together with their vigor and ethylene production. Khan (1994b) reported that both naturally and artificially aged 'Mesa 659' seeds produced less ethylene and germinated less at 25°C than nonaged seeds. With age, the mean time to radicle protrusion also increased. Khan (1994b) also reported that aged seeds had a lower ability to convert exogenous ACC to ethylene. Nascimento et al. (2004b) observed that 'Everglades' seeds artificially aged for three or five days had lower germination at 35°C than nonaged seeds, 73% and 44%, respectively, as compared to 100%. The same author also described a significant decrease in ethylene production during germination at both 20°C and 35°C following artificial aging for one, three, or five days.

The importance of ethylene for lettuce germination under stressful conditions has been attributed to both a decrease in ethylene production and an increase in ethylene requirements. At inhibitory temperatures, ethylene production may either increase or decrease, depending on the genotype (Nascimento et al., 2000). Seeds from thermosensitive genotypes, 'Dark Green Boston' and 'Valmaine', produced less ethylene at 35°C than at 20°C. Contrary, in thermotolerant 'Floricos', 'Everglades' and 'PI 251245' seeds, ethylene production increased at 35°C. The amount of ethylene produced correlated with germination for all cultivars. Prusinski and Khan (1990) reported that slit lettuce seeds from nine cultivars produced more ethylene at both 32°C and 35°C than intact seeds. These authors hypothesized that the seed coat might create a hypoxic environment unfavorable for conversion of ACC to ethylene. Huang and Khan (1992) reported that preconditioning of lettuce seeds led to ACC detection prior to their germination at 35°C and to high germination. The seeds were preconditioned by being imbibed in water or test solution in the presence of solid carrier, Micro-Cel E, for four to 10 hours at 7°C and 28% relative humidity. Non-conditioned seeds did not germinate at 35°C and ACC was not detectable. Both Abeles (1986) and Nascimento et al. (2004a) reported that ethylene action inhibitors (MCEB, STS) had greater effect on germination at supraoptimal temperature that at optimal temperature. The authors hypothesized that more ethylene might be required for germination at supraoptimal temperature. It is possible that both processes, a decrease in ethylene production and an increase in requirements for ethylene, occur simultaneously under inhibitory conditions.

Several mechanisms have been proposed to explain the promotive effect of ethylene on germination. Abeles (1986) hypothesized that ethylene might increase the

growth potential of the lettuce embryo. He reported that germination of isolated 'Grand Rapids' embryos decreased in increasing mannitol concentrations and that the application of ethylene increased germination. The same author also described inhibition of lettuce germination at 25°C under hyperbaric (five bars) conditions that was reversed by application of the ethylene-releasing compound ethephon. The ability of ethylene to promote radicle protrusion could be explained by its ability to promote hypocotyl expansion (Abeles, 1986). Lettuce seedlings growing at 30°C in the presence of ethylene had a hypocotyl diameter of 0.99 mm as compared to 0.76 mm in air.

Ethylene has also been hypothesized to participate in regulation of endosperm weakening. Abeles (1986) reported that ethylene reduced the structural integrity of lettuce endosperms. Nascimento et al. (2000) correlated the activity of endo-beta-mannanase with ethylene production under different experimental conditions. Thermotolerant lettuce genotypes ('Floricos', 'Everglades', and 'PI 251245') had greater endo-beta-mannanase activity and produced more ethylene at 20°C and 35°C than thermosensitive genotypes ('Dark Green Boston' and 'Valmaine'). When 'Everglades' seeds were imbibed at 35°C in 20 mM STS, they produced significantly less ethylene than seeds imbibed in water due to possible disruption of the auto-stimulation of ethylene biosynthesis by the hormone itself, and did not have detectable endo-beta-mannanase activity.

At the molecular level, there is limited information about the mechanism of action of ethylene on germination or the ability of stressful conditions to reduce ethylene production or suppress ethylene perception. It is possible that the activities of both ACC synthase and ACC oxidase, key enzymes in ethylene biosynthesis, are reduced under

stressful conditions. Khan and Huang (1988) reported that the conversion of ACC to ethylene was reduced under saline conditions. Huang and Khan (1992) observed better germination at 35°C of preconditioned 'Mesa 659' seeds in ethephon than in ACC.

Effects of Ethylene on Germination of Other Plant Species

The presence of ethylene is a prerequisite for germination in many plant species. Bleecker et al. (1988) observed lower germination in ethylene-insensitive *Arabidopsis* seeds as compared to wild-type seeds. Ethylene production increased during imbibition reaching a peak at time of radicle protrusion in white lupin (Bekman et al., 2000), chickpea (Gomez-Jimenez et al., 2001), and olive (Rinaldi, 2000). Application of inhibitors of either ethylene production (AVG) or action (NBD) decreased or completely inhibited germination (Rinaldi, 2000; Gomez-Jimenez et al., 2001). The inhibitory effect was partially or completely reversed by application of ACC (Rinaldi, 2000; Gomez-Jimenez et al., 2001). Ethylene production was localized in the embryonic axes for both pea (Petruzzelli et al., 2000) and chickpea (Gomez-Jimenez et al., 2001).

The involvement of ethylene in germination under stressful conditions in plant species other than lettuce has been extensively studied. Some seeds (sunflower, *Amaranthus retroflexus*) germinate well at high temperatures (35-40°C) but do not germinate if temperatures are reduced below the optimal. Chojnowski et al. (1997) reported that the amount of ethylene produced by sunflower seeds was related to their ability to germinate at suboptimal temperature. Kepczynski et al. (1996) observed an increase in germination of *Amaranthus retroflexus* seeds at suboptimal temperatures after application of 10^{-4} to 10^{-6} M ethephon. Ethylene can also stimulate germination under salt

stress. Khan and Ungar (2002) reported that application of ethylene stimulated germination of *Zygophyllum simplex* seeds at increasing salt concentrations. Gul et al. (1998) reported that application of ethephon increased germination of *Allenrolfea occidentalis* seeds at salt concentrations up to 600 mM NaCl. The authors also observed a dependence of the ethephon action on imbibition temperature, with temperatures of 20° - 30°C correlating with the strongest ethephon effect. Gomez-Jimenez et al. (2001) reported inhibition of ethylene synthesis under unfavorable germination conditions (application of ABA or low water potential).

Ethylene can break seed dormancy in certain seeds. Kepczynski et al. (2003a) reported that application of ethylene circumvented the requirement for dry storage and allowed dormant *Amaranthus retroflexus* seeds to germinate at 30°C. When NBD was applied simultaneously with ethylene, the promotive effect of ethylene decreased in a concentration-dependent manner. In a related species, *Amaranthus caudatus*, both ethylene and ACC removed secondary dormancy, imposed by supraoptimal temperature. Again, the dormancy breaking effect of these compounds was reversed upon application of NBD. Barros et al. (1998) reported partial breaking of dormancy of *Stylosanthes humilis* seeds in the presence of ACC. Macchia et al. (2001) reported that application of ethylene increased the effect of other dormancy-breaking treatments like prechilling in *Echinacea angustifolia*. In certain species like cocklebur, dormancy has been related to decreased ethylene production (Satoh and Esashi, 1983). Kepczynski et al. (2003a) reported that nondormant *Amaranthus retroflexus* seeds did not produce more ethylene during imbibition than dormant seeds. Since these authors observed an increase in ACC

oxidase activity *in vivo* prior to germination, they hypothesized that dormancy maintenance might be due to insufficient ACC content.

Ethylene production has been associated with seed vigor in plant species other than lettuce. Khan (1994b) reported that artificially aged cabbage, tomato, snap bean and sweet corn seeds produced less ethylene with or without application of ACC than non-aged seeds. Siriwitayawan et al. (2003) reported that both naturally and artificially aged tomato and sweet corn seeds with decreased vigor but high germinability produced significantly lower ethylene quantities than non-aged seeds. It is possible that seed lots with maximal germination under optimal conditions still differ in their ability to produce ethylene and, consequently, to germinate under inhibitory conditions. These authors hypothesized that reduced ethylene production in aged seeds was due to reduced conversion of ACC to ethylene. They reported no correlation between ACC content and ethylene production in non-aged or aged tomato and sweet corn seeds. The reduced ability for ACC conversion can be a nonspecific process, one of the signs of decreased protein and RNA synthesis associated with aging.

Several mechanisms have been proposed to explain the promotive effect of ethylene on germination in different plant species. Matilla (2000) hypothesized that ethylene might participate in the accumulation of amino acids in cocklebur, a process essential for development of osmotic potential. Maruyama et al. (1997) reported ethylene-induced accumulation of asparagine and aspartate in cocklebur seeds.

The second proposed mechanism is that ethylene stimulates activity of enzymes involved in weakening of tissues restricting germination. Leubner-Metzger et al. (1998) reported that class I β-1, 3-glucanase was involved in rupturing of endosperm in tobacco

seeds and was expressed exclusively in micropylar endosperm prior to germination. Application of 100 ppm ethylene stimulated transcription of the glucanase gene, the activity of the protein and the expression of fusion constructs containing the glucanase promoter. When inhibitors of ethylene production (1 mM AVG) and action (50 ppm NBD) were applied, the glucanase activity was lower than in control seeds. The presence of a nucleotide sequence, known as the ethylene responsive element (ERE) in the glucanase promoter, was also reported (Leubner-Metzger et al., 1998). Petruzzelli et al. (1999) reported the presence of ethylene-inducible β-1, 3-glucanase in pea seeds. Ethylene stimulates enzyme activity even in some monocot species like *Amaranthus caudatus*. Bialecka and Kepczynski (2003) reported that ethephon-treated *Amaranthus caudatus* seeds had 1.5- to 2- fold higher α-amylase activity than control seeds, and their germination was higher.

Another possible explanation for the promotive effect of ethylene is that ethylene antagonizes the effect of some germination-inhibiting factors (possibly ABA). An antagonism between ethylene and ABA has been reported for *Arabidopsis thaliana* seeds (Beaudoin et al.,2000; Ghassemian et al., 2000) Application of ethylene was reported to induce precocious germination in *Phaeolus vulgaris* (Matilla, 2000).

No attempt has been made for the creation of transgenic plants with altered ethylene perception or production in order to specifically study the effect of ethylene on germination. Several observations have been made in the course of studies of mutant ethylene-insensitive seeds. Bleecker et al. (1988) reported that ethylene-insensitive *Arabidopsis thaliana* seeds had lower germination than wild-type seeds. Their germination was improved following application of gibberellins. Both Beaudoin et al.

(2000) and Ghassemian et al. (2000) reported that *Arabidopsis thaliana* seeds with altered ethylene perception had altered sensitivity to ABA and altered dormancy. *Arabidopsis thaliana ein1* (also ethylene insensitive) seeds were unable to germinate immediately after harvest and required at least four days of chilling to reach 100% germination (Ghassemian et al., 2000). Freshly harvested wild-type *Arabidopsis* seeds germinated at 100%. *Ein2* seeds (also ethylene insensitive) demonstrated increased and age-dependent sensitivity (as compared to wild-type seeds) to exogenous ABA. Contrary, constitutive triple response *Arabidopsis thaliana* seeds (*ctr1*) were less sensitive to exogenous ABA than wild-type seeds (Beaudoin et al., 2000). They germinated only slighter better than wild-type seeds immediately after harvest. It appears that the level of endogenous ethylene or ethylene precursors in wild-type seeds is at, or close to, the saturation point for action on seed dormancy. Similar to *Arabidopsis*, *Petunia hybrida* ethylene insensitive seeds also had lower germination than wild -type seeds (Barry, 2004). Their germination was improved by application of GA. However, germination of tomato ethylene insensitive seeds was not reduced. This could be explained by different requirements for ethylene by different plant species.

The molecular mechanisms of ethylene action during germination are unknown. Germination-specific isogenes for the two key enzymes in ethylene biosynthesis, ACC synthase and ACC oxidase have been reported. Bekman et al. (2000) described two ACC synthase mRNA transcripts that were up-regulated during germination of white lupin seeds and non-affected by ABA treatment. Petruzzelli et al. (2000) and Gomez-Jimenez al. (2001) reported the existence of germination-specific ACC oxidase genes in pea and chickpea seeds, respectively. Expression of these genes began during early imbibition,

increased throughout germination and reached maximum levels at the time of radicle protrusion. Since treatment with NBD reduced both ACC oxidase expression and activity in pea seeds, increasing the ACC content, it was concluded that this activity was at least partially stimulated through a positive feedback mechanism by ethylene production. There is little information about germination-specific ethylene perception. Lashbrook et al. (1998) reported that expression of one of the tomato ethylene receptor genes (*LeETR2*) was induced prior to germination. Leubner-Metzger et al. (1998) reported that an ERE was located in the promoter region of the ethylene-induced 1, 3-β-glucanase gene. Deletion of the -1193 to -1452 region of the glucanase gene, contain the ERE, resulted in the inability of ethylene to induce enzyme expression. Leubner-Metzger et al. (1998) also described a class of ethylene-responsive element binding proteins (EREBP-3) that was induced earlier in ethylene-treated tobacco seeds and was inhibited by ABA. This group hypothesized that EREBP-3 was a transcription factor, important for the ethylene-dependent, high level transcription of the glucanase gene.

Interactions Between Ethylene and Other Plant Hormones During Germination

Interactions between ethylene and ABA have been reported during seed development and germination of several plant species. Beaudoin et al. (2000) and Ghassemian et al. (2000) reported that ethylene-insensitive *Arabidopsis thaliana* seeds were more sensitive and seeds possessing constitutive triple response less sensitive than wild-type seeds to exogenously supplied ABA. The enhanced sensitivity to ABA depended on the age of the seeds and decreased with age. Since seeds with ethylene insensitivity conferred by mutations in different genes (*ein* or *etr*) demonstrated enhanced

sensitivity to ABA, Ghassemian et al. (2000) concluded that functional ethylene signaling was required for correct ABA sensitivity. Barros and Delatorre (1998) reported that ACC-mediated dormancy breaking in *Stylosantes humilis* seeds was inhibited by application of ABA. Interaction between ABA and ethylene has been reported during release of both primary dormancy in *Amaranthus retroflexus* (Kepczynski et al. 2003a) and secondary dormancy in *Amaranthus caudatus* (Kepczynski et al. 2003b). In *Amaranthus retroflexus*, ABA decreased the ethylene effect indicating an increased requirement for exogenous ethylene.

There is little information about the mechanism of interaction between ethylene and ABA. Gomez-Jimenez et al. (2001) reported that germination of chick-pea seeds in 50 μM ABA led to a decrease in ACC content (suggesting inhibition of ACC synthase activity) and altered the time course of ACC oxidase activity. Bekman et al. (2000) observed down-regulation of transcription of ACC synthase in ABA treated plants. The ABA-mediated down-regulation might be due to the presence of an ABA-responsive element (ABRE) in the promoter of this gene. Leubner-Metzger et al. (1998) reported that application of ABA decreased and delayed accumulation of germination-induced EREBP-3 mRNA transcripts. It is possible that there are several mechanisms involved in the ABA-ethylene interaction.

Interaction between ethylene and GAs and/or light has also been reported. An interaction between ethylene and GA was observed during germination of *Amaranthus retroflexus* (Kepczynski et al., 2003a). Application of GA_3 only partially removed *A. retroflexus* dormancy but enhanced the stimulatory effect of ethylene. When ethylene synthesis was blocked, the promotive effect of GA_3 on dormancy breaking was

decreased. It appears that ethylene biosynthesis is required for GA_3 action during dormancy release. Germination of some GA-deficient seeds (*Arabidopsis thaliana*) but not others (tomato) can be rescued with application of ethylene in light (Hilhorst and Karssen, 1992). Germination of some ethylene insensitive seeds (*Arabidopsis*, petunia) can be enhanced by application of GA (Bleecker et al., 1988). Bessler et al. (1998) hypothesized that light stimulated ethylene production in *Tillandsia usneoides* through increasing the amount of available ACC or enhancing ethylene perception.

The interaction between ethylene and cytokinins has been studied in several plant species. Khan and Prusinski (1989) reported that relief of dormancy by application of cytokinins in peanut seeds was accompanied by enhanced ethylene production. Release of dormancy in cocklebur and Indian rice grass seeds was synergistically or additively enhanced by a combination of kinetin and Ethrel. Kinetin and Ethrel both promoted lettuce germination, but acted synergistically only when the seeds were germinated under inhibitory conditions (salt stress, supraoptimal temperature) (Khan and Huang, 1988; Khan and Prusinski, 1989). Cytokinins and ethylene may interact on several different levels. Khan and Prusinski (1989) reported that application of 1 mM Co^{2+} (an inhibitor of the ACC to ethylene conversion) to 'Mesa 659' lettuce seeds negated the promotive effect of a kinetin and ACC combination on germination. This suggested that cytokinins may enhance the activity of ACC oxidase. Huang and Khan (1992) reported further stimulation of germination of 'Mesa 659' seeds under saline conditions when kinetin was applied to saturating levels of ACC. These authors also reported that simultaneous application of kinetin and AVG lowered the promotive effect of kinetin on germination and decreased ethylene production. It appears that cytokinins may also stimulate activity

of ACC synthase. Another possibility is that cytokinins act through enhancing ethylene perception.

In conclusion, the hormonal regulation of lettuce germination has been extensively studied and the positive role of gibberellins and cytokinins and the negative effect of ABA have been recognized. The importance of ethylene for lettuce germination, especially under inhibitory conditions, has been accepted. However, the exact mechanism of ethylene action is unknown.

Mechanisms of Lettuce Germination

Embryonic Growth Potential

Lettuce embryos need to develop sufficient growth potential in order for the seeds to germinate. Growth potential is determined as the osmotic potential required to achieve 50% germination (Scheibe and Lang, 1965). The osmotic potential of lettuce embryos is measured by placing embryos in mannitol solutions with pre-determined molarity and observing their germination (Takeba, 1980). The growth potential of 'New York' lettuce seeds was 0.44 M in dark at 27°C compared to 0.24M at a supraoptimal temperature (35°C) (Takeba and Matsubara, 1979). Application of red-light improved lettuce germination and increased growth potential at 27°C. However, the light treatment did not affect osmotic potential at 35°C. When 'New York' seeds were imbibed in 1 mM GA, they germinated 92% as compared to 8% for non-treated seeds at 27°C in dark. GA-treated seeds had a growth potential of 0.54 M compared to 0.44 M for nontreated seeds (Takeba, 1984).

The increase in osmotic potential in lettuce seeds has been associated with accumulation of free amino acids, especially glutamate and glutamine. Takeba (1980) observed that imbibition of lettuce seeds was accompanied by an increase in the amount of free amino acids. At 27°C, in dark, the amount of free amino acids was 30 μmol/axes from three grams of dry seeds. Germination promoting treatments, like application of 1 mM GA increased amino acids to 45 μmol/axes (Takeba, 1980). The difference of 15 μmol/axes accounted for a difference of 0.2 M in growth potential. Using chromatographic methods, Takeba (1980) discovered that the major amino acids accumulated were glutamate and glutamine.

Glutamine synthetase is the enzyme involved in synthesis of glutamate and glutamine in seeds. Takeba (1983a, 1984) reported that treatments promoting lettuce germination and increasing growth potential also enhanced glutamine synthetase activity. Red-light irradiation increased the activity of glutamine synthetase from 0.22 A540/0.1g dry seeds to 0.41 in 'New York' seeds imbibed at 27°C (Takeba, 1983a). Since this increase was observed eight hours after the start of imbibition and in osmotically-inhibited seeds, it was a prerequisite for germination, not a process accompanying it. Takeba (1984) reported that application of 1 mM GA also enhanced glutamine synthetase activity at 27°C in both light and dark, to 1.82 and 1.42, respectively (0.92 for the dark control). Simultaneously, germination of 'New York' seeds was significantly increased, to 98 and 85%, respectively (8% for the control). Application of cyclohexamide (a protein synthesis inhibitor) completely suppressed glutamine synthetase activity and germination, while application of actinomycin D (RNA synthesis inhibitor) did not have an effect on glutamine synthetase. It appears that this enzyme has to be synthesized *de*

novo on preexisting mRNA during imbibition. Takeba (1984a) applied a specific inhibitor of glutamine synthetase, L-methionine-DL-sulfoximine to 'New York' lettuce seeds and observed a simultaneous decrease in enzyme activity and inhibition of both germination and the GA promotive effect. It is very probable that GA partially promotes lettuce germination through enhancing glutamine synthetase activity.

Though increasing internal osmotic potential of the seed seems to be required for lettuce germination under optimal germination conditions, it does not seem to be totally responsible for germination under inhibitory conditions. Takeba (1983a) reported that imbibition of 'New York' seeds at 35°C decreased glutamine synthetase activity and inhibited germination. Neither red-light nor GA reversed the effect of supraoptimal temperature.

The ability of lettuce embryos to germinate (growth potential) can be modified not only through solute accumulation but also through change in pressure potential (the impact of the rigid plant cell walls on water movement) via increased cell wall loosening. Carpita et al. (1979) reported that red-light treated lettuce seeds had increased secretion of H^+ and increased uptake of K^+ and Na^+ compared to far-red-light treated seeds. These authors hypothesized that the altered cation transport was a result of action of a red-light stimulated proton pump. Cooley et al. (1999) reported a GA-stimulated H^+-ATPase that was up-regulated in tomato seeds prior to radicle protrusion.

Endosperm Weakening

For many years lettuce germination has been suggested to be result of an increase in growth potential and some other factor. During the 1960s it was suggested that

endosperm weakening was also a prerequisite for lettuce germination. Pavlista and Haber (1970) reported that application of increasing concentrations of sodium dichloroisocyannurate (chlorine-releasing compound) to 'New York' lettuce seeds led to a decrease in the percent of normally germinated seeds and an increase in seeds with embryo expansion without protrusion (embryo buckling) and atypically germinated seeds. These authors concluded that the chlorine-releasing compound inhibited endosperm weakening which should facilitate radicle protrusion. Other evidence supporting the idea that endosperm tissue restricts germination in lettuce comes from the observations of Jones (1974). This author reported that endosperm cell walls were modified compared to normal plant cell walls. Lettuce endosperm cell walls had many peg-like projections that came from all four walls, and often fused to form bridges across the cytoplasm. He hypothesized that these protuberances might contribute to the structural rigidity of the endosperm.

The suggestion that endosperm weakening is a prerequisite for lettuce germination is based to a large extent on observations of the change in strength of lettuce endosperm during germination. Tao and Khan (1979) used an Instron Universal testing machine to examine the contribution of the lettuce pericarp and endosperm to the restriction of radicle protrusion. They measured the force required to puncture intact 'Grand Rapids' seed, seed minus pericarp, and excised embryo (puncture test), and reported that the endosperm strength represented about 60% of the total force required to puncture the seed. In addition, only the force required to puncture the endosperm was significantly lower under germination-favoring conditions (light at 25°C and dark plus GA_3 at 25°C) than under germination-inhibitory conditions (dark at 25°C). Lower

endosperm strength was observed in seeds imbibed for 6, 12, and 24 hours that suggested that endosperm weakening was a prerequisite for radicle protrusion. Sung et al. (1998) measured the force required to puncture intact seeds from three thermotolerant and two thermosensitive lettuce genotypes. They reported that more force was required to penetrate both intact seeds and endosperms of thermosensitive than thermotolerant genotypes during imbibition at either 24°C or 33°C in light. Primed seeds from both thermotolerant and thermosensitive genotypes required less penetration force than nonprimed seeds. However, primed seeds from thermosensitive genotypes still required more force than those from thermotolerant genotypes.

Changes in cell anatomy of the micropylar endosperm or changes in the endosperm as a whole have been associated with lettuce germination. Pavlista and Valdovinos (1978) using conventional scanning electron microscopy observed the appearance of cracks and pits on the surface of the endosperm of 'Grand Rapids' lettuce seeds. The cracks and pits appeared after 8 hours of imbibition (20°C in dark or light) and became more extensive in 9- and 10-hour imbibed seeds. In many 10- to 14-hr imbibed seeds, the seed tips were partially collapsed and had cell separation. In addition, some seeds imbibed for 10 or more hours had an opening or large break (fissure) in the micropylar part of the endosperm. All surface alterations were confined to the micropylar part of the endosperm. Regardless of germination conditions (continuous white light or dark/ 20°, 24° or 35°C), the percent of seeds with endosperm alterations correlated with the percent germinated seeds. Guedes et al. (1981) also observed morphological changes in endosperms of primed 'Minetto' lettuce seeds prior to germination.

Differences in the internal anatomy of cells in micropylar and lateral regions of the endosperm have been observed prior to and after lettuce germination. Georghiou et al. (1983) reported that 'Grand Rapids' seeds imbibed for one hour at 25°C in dark had micropylar cells with dense cytoplasm, few vacuoles and abundant storage material. After 12 hours of imbibition, the appearance of the micropylar cells changed. A part of the storage material, especially proteins, was mobilized and the protein bodies developed into vacuoles. Since imbibition of the seeds under germination-inhibitory conditions like continuous far-red light, supraoptimal temperature (35°C) or osmoticum (0.35 M mannitol) did not lead to any changes in cell anatomy; it appeared that the changes were associated with germination. Psaras et al. (1981) and Psaras and Georghiou (1983) reported that in light-requiring 'Grand Rapids' seed lots the anatomical alterations were observed only after application of red-light or GA, but not in dark. In all cases, the changes were observed only in micropylar cells. The lateral cells were unchanged prior to and immediately after germination. Psaras and Paragamian (1984) reported that identical anatomical alterations were observed in micropylar cells of isolated endosperms, incubated in GA. Interestingly, these authors observed changes only if the endosperms were isolated from imbibed seeds intact for nine hours. It appeared that there was a need for a stimulus to come from the embryo and to make the endosperm responsive to GA. Alternatively, the endosperm cells needed to be fully imbibed in order to respond to exogenous stimuli. Psaras (1984) reported that the differences in appearance of micropylar and lateral cells were preserved even 48 hours after radicle protrusion. Micropylar cells were rounded, with a central vacuole. Lateral cells had disintegrated

protoplasts and weakened cell walls. The author hypothesized that the two cell types had different functions during germination and post-germinative mobilization.

Sung (1996) observed both changes in the surface appearance of the endosperm and in the internal anatomy of micropylar cells prior to germination. A crack was evident on one side of the endosperm in both primed and nonprimed 'Dark Green Boston' and 'Everglades' seeds. The crack was more prominent in primed seeds and appeared earlier in primed 'Everglades' seeds than ' Dark Green Boston' seeds. More prominent cracks were also observed in both 'Everglades' and 'Dark Green Boston' seeds matured at 30°/20°C as compared to 20°/10°C. In some cases, the endosperm was separated from the integument. Seeds matured at 30°/20°C also had a lower number of cells in the micropylar endosperm than seeds matured at 20°/10°C. In all seeds, the micropylar endosperm consisted of strongly vacuolated cells, sometimes with protein bodies inside the vacuoles. In some instances, the micropylar cell walls were broken. Contrary, the lateral endospermic cells had loose cytoplasm.

Some authors did not observe changes in the endosperm of lettuce seeds prior to germination and concluded that endosperm weakening was not a prerequisite for germination. Using low-temperature scanning electron microscopy, Nijsse et al. (1998) did not observe any cracks on the surface of 'Little Gem' lettuce seeds, imbibed at 20°C or 25°C in 1-hr white-light irradiation or far-red irradiation. The authors concluded that the cracks observed by Pavlista and Valdovinos (1978) were artifacts, result of the electron microscopy technique used by these authors. Another explanation for the discrepancy between the results of Pavlista and Valdovinos and Nijsse may be the use of different lettuce cultivars with different germination patterns.

The different appearance of cells in the micropylar region of the endosperm suggests that this region may have a different function than the rest of the endosperm. Takeba (1980) hypothesized that accumulation of free amino acids was a prerequisite for radicle protrusion in lettuce. It is possible that the degradation of protein bodies in the micropylar cells provides amino acids necessary for development of growth potential. Another possibility is that the micropylar cells secret enzymes, participating in cell wall weakening. Jones (1974) reported that endosperm cells possessed all the cytological prerequisites for protein synthesis. The protein bodies might provide amino acids or small peptides for the synthesis of new enzymes. The finding that the digestion of lettuce endosperm proceeded from the plasmalemma of each cell outward and that the outer wall of the endosperm cells next to the integument was not digested supported the hypothesis that the endosperm itself produced the enzymes weakening it. Similar cell wall breakdown exists in barley aleuronic layers (Taiz and Jones, 1970).

Endosperm weakening has been reported to be a prerequisite for germination in several other plant species. Downie et al. (1999) observed that ABA-deficient (*sit (w)*) tomato seeds had internal free space at completion of seed development. These seeds also germinated more rapidly than wild-type seeds under variety of germination conditions, including imbibition in water, ABA or under far-red light irradiation. Such a free space was also observed in wild-type tomato seeds after priming. Toorop et al. (2000) reported that tomato endosperm weakening proceeded in two phases. The first phase was dependent on the presence of endo-beta-mannanase and not inhibited by ABA. The second phase was not dependent on endo-beta-mannanase and inhibited by ABA. The same researchers also observed a sharp border between the micropylar and lateral regions

of tomato endosperm that suggested that the cells in the two regions were structurally and functionally different. Sanchez et al. (1997) reported erosion in the micropylar endosperm of *Datura ferox* seeds in response to red-light irradiation. Restriction of embryo growth by tissues surrounding the embryo had been reported for yellow cedar. Ren and Kermode (1999) observed a decrease of the mechanical strength of the micropylar region during germination of yellow cedar seeds.

The Role of Endo-Beta-Mannanase (EBM) in Endosperm Weakening and Germination

Endo-(1, 4)-beta-mannanase (EC 3.2.1.78), a cell wall weakening enzyme, is emerging as one of the most important factors regulating germination under optimal and germination-inhibiting conditions for many plant species that contain endosperm. Its participation in endosperm weakening prior to germination has been demonstrated for tomato, *Datura ferox*, lettuce, *Picea glauca* (Nonogaki et al., 2000; Sanchez et al., 1997; Nascimento et al., 2000; Downie et al., 1997) and has been hypothesized for pepper (Watkins et al., 1985).

Lettuce Endo-Beta-Mannanase (EBM)

The activity of lettuce EBM was first reported as part of post-germinative mobilization of stored reserves from the endosperm (Halmer et al., 1976). The authors observed an increase in EBM four to five hours after the start of imbibition of 'Grand Rapids' seeds at 23°C in dark or light that proceeded almost linearly up to 33 hours. The EBM activity always correlated with percent germination. The authors concluded that the higher enzyme activity observed during conditions favoring germination was due to a

higher percentage of germinated seeds in the seed batches used for enzyme extraction. Application of red-light or 0.29 mM GA strongly increased both germination and EBM activity in 'Grand Rapids' seeds. If far red-light or 0.03 mM ABA were applied, germination was lower than that in control conditions as was EBM activity. Leung et al. (1979) hypothesized that the role of EBM in post-germinative mobilization of storage reserves was to degradate mannan polymers to oligomannans. These oligomannans were transported to the cotyledons where they were further degraded to monomers with the help of β-mannosidase. Halmer and Bewley (1979) reported that EBM was also produced by isolated endosperms of 'Grand Rapids' seeds. EBM production in isolated endosperms begun approximately 9 hours after the start of imbibition (5 hours after separation of embryos and endosperms). In comparison, EBM production in intact seeds began about 14 hours after start of imbibition. However, in both cases, the maximum production was observed about 30 hours after sowing. There was no need for any external stimulus (light or GA) in order for EBM to be produced.

Early studies of lettuce endo-beta-mannanase (Halmer et al., 1976; Halmer and Bewley, 1979) only reported production of the enzyme after completion of germination. However, Halmer et al., (1976) observed that application of 100 μg/ml cyclohexamide at different times during lettuce imbibition led to a strong decrease in EBM production and germination. This suggested a correlation between EBM and germination. When lettuce seeds were imbibed in combinations of GA and ABA or GA, ABA and BA, some EBM activity was observed in nongerminated seeds. The same authors also reported that dry and nongerminated 'Grand Rapids' seeds exhibited low (basal) EBM activity. Since anatomical changes were observed only in the micropylar region of the endosperm prior

to germination, these authors concluded that it might be possible that this low activity was sufficient for cell wall weakening prior to germination.

The first strong evidence for endo-beta-mannanase activity prior to germination came from the work of Dutta et al. (1997). These authors reported that endosperm cell walls of 'Pacific' lettuce seeds imbibed at 25°C or 32°C in dark exhibited cell wall autolysis. The rate of autolysis increased up to 14 hours from the start of imbibition and the amount of released monomers also increased with time. Since the rate of carbohydrate release was dependent on both temperature and pH, it was very probable that it was mediated by enzymatic action. Interestingly, the rate of hydrolysis of micropylar walls was about three times higher than that of walls prepared from lateral endosperm. Endospermic walls prepared from seeds imbibed at thermoinhibitory conditions (32°C) exhibited slower autolysis than walls of seeds imbibed at 25°C. However, if seeds were imbibed at 32°C in GA or kinetin, germination was improved and the rate of wall autololysis was increased. Contrary, if seeds were imbibed in 100 μM ABA at 25°C, they did not germinate and the rate of autolysis was significantly lower. Dutta et al. (1997) further decided to extract and characterize the enzyme activity responsible for the cell wall autolysis in lettuce endosperms. The authors reported that at least a component of this activity was attributed to EBM. The decrease in polymer size of locust bean gum (LBG) but lack of monomer release indicated an endo-type of activity. The LBG-hydrolyzing activity in cell wall extracts had a pH optimum of 5.0 that coincided with the pH optimum of lettuce cell wall autolysis. In addition, the cell wall extracts were less active in mediating the release of reducing sugars from LBG under the same imbibition conditions (supraoptimal temperature (32°C) or ABA) that led to decreased cell wall

autolysis. Dutta et al. (1997) observed the endo-hydrolyzing activity in extracts of two different lettuce cultivars, 'Pacific' and 'Grand Rapids'.

Strong support for the importance of endo-beta-mannanase for endosperm weakening and subsequent germination came from the work of Nascimento et al. (2000,2001). This group correlated EBM activity with germination under germination promoting- and inhibiting–conditions in several lettuce cultivars. Nascimento et al. (2000) examined EBM activity 1 hour prior to, immediately after radicle protrusion, and 24 hours after the start of imbibition in several thermosensitive and thermotolerant genotypes. They reported that seeds from the thermotolerant genotype 'Everglades' produced more EBM at 1hour pre-germination , 1 hour post-germination, and 24 hours than did seeds from the thermosensitive genotypes ' Dark Green Boston' and 'Valmaine' at both 20ºC and 35°C. Sung (1996) observed anatomical changes and improved germination in seeds matured at 30°/20ºC but not in those matured at 20°/10ºC. Nascimento et al. (2000) observed higher EBM activity in both 'Dark Green Boston' and 'Everglades' seeds matured at 30°/20ºC than at seeds matured at 20°/10ºC at both 1 hour pre-germination and 1 hour post-germination at both 20ºC and 35ºC. In all seeds, EBM activity was greater at 20ºC than at 35ºC. 'Everglades' seeds or seeds matured at 30°/20ºC were able to produce detectable amounts of EBM prior to germination at 35ºC while 'Dark Green Boston' and 20°/10ºC seeds were not able to do so. It appears that the inability of lettuce seeds to germinate at supraoptimal temperature is related to lack of sufficient activity of EBM for endosperm wall loosening. Nascimento et al., (2000) also reported that EBM activity was significantly higher in micropylar endosperm than in lateral, which was in support of the hypothesis for participation of EBM in endosperm

weakening. Interestingly, 'Everglades' seeds produced more EBM at 27.5°C than at 20°C but less at 35°C than at 20°C. This points toward general stimulation of seed metabolism and protein synthesis by slightly increased temperature followed by thermoinhibition at 35°C. 'Everglades' seeds also produced more EBM during imbibition in light than in dark.

Priming that alleviates lettuce thermoinhibition also leads to increased detectable EBM activity. Nascimento et al. (2001) reported significantly higher EBM activity in both 'Dark Green Boston' and 'Everglades' seeds immediately after priming and prior to germination at both 20°C and 35°C. Primed seeds from both genotypes not only germinated significantly better at 35°C (100% germination for primed DGB at 35°C compared to 4% for nonprimed) but also germinated more rapidly (germination rate of 4 hours for primed DGB seeds at 35°C compared to 18 hours for nonprimed). Priming did not alleviate the differences in EBM activity between thermosensitive and thermotolerant seeds. In contrast, EBM activity of 'Everglades' seeds after 48 hours of priming was 60 times higher than that in 'Dark Green Boston' seeds. High EBM activity that accounted for about 50% of the activity in the whole seed was observed in the micropylar region of the endosperm following priming. Drying of primed seeds back to original moisture content did not decrease EBM activity. Nonogaki et al. (1992) also observed that about 70% of the EBM activity was retained after drying of tomato seeds. Since Sung et al. (1998) has observed that priming enhances endosperm weakening and germination, a correlation appears to exist between endosperm weakening, germination, and EBM activity.

Seed aging (natural or artificial) has been reported to decrease seed vigor in different plant species and the ability of the seed to germinate under inhibitory conditions (Khan, 1994b). Nascimento et al. (2004b) observed decreased EBM activity in artificially aged 'Everglades' seeds at both 20ºC and 35ºC, in both nongerminated and germinated seeds. The decrease of EBM activity was proportional to the duration of the accelerated aging treatment. It is possible that the aging process leads to an unspecific decrease in protein synthesis, which, in its turn, leads to lower EBM activity and lower germination.

The discrepancies between the different researchers regarding EBM activity prior to germination can be explained by differences in the experimental procedures and plant material used. Dutta et al. (1997) used a reducing sugar release assay and Halmer et al. (1976) used a viscometric assay. Though a viscometric assay is considered to be more sensitive than a reducing sugar assay, Dutta et al. (1997) used 6000 endosperms per treatment as compared to 200 used by Halmer et al. (1976). Nascimento et al. (2000, 2001) used a single seed gel-diffusion assay as adapted from Still et al.(1997) that allows detection of EBM activity in individual seeds or seed parts. It is possible that the use of this technique allowed detection of minor differences in the very low activity prior to germination observed before by Halmer et al. (1976). However, all the discrepancies cannot be explained by use of different experimental procedures since Nonogaki and Morohashi (1999) did not observe EBM activity prior to germination in lettuce seeds using the single seed gel-diffusion assay. However, these authors used a thermosensitive lettuce cultivar 'Green Wrap' and imbibition conditions of 28ºC in dark that were, most probably, unfavorable for EBM production.

Despite the characterization of lettuce endo-beta-mannanase activity, little is known about the metabolic requirements for its production and about its protein and gene structure. Dulson and Bewley (1989) and Halmer (1989) reported that EBM is synthesized *de novo* by the endosperm cells. Dulson and Bewley (1989) observed that concentrations of cyclohexamide as low as 0.1 mM inhibited the production of EBM in isolated endosperms suggesting that the production of this enzyme was only a minor part of the endosperm protein synthesis. Halmer (1989) further reported that EBM appeared to be produced continuously since addition of cyclohexamide at different times during imbibition decreased its quantity. The same author also reported that the mRNA for EBM seemed to be synthesized also *de novo* since the production of EBM was affected following imbibition in the presence of RNA synthesis inhibitors. However, these inhibitors were only effective if present during the first hours of imbibition suggesting that there was a separation in time of EBM RNA and protein synthesis. Halmer (1989) did electrophoretic characterization of lettuce EBM. He established the molecular weight of EBM to be 46 kD and the protein to be present as three bands under native gel conditions. Nonogaki and Morohashi (1999) also observed three bands corresponding to lettuce EBM in native gels. If lettuce extracts were subjected to native protein electrophoresis followed by denaturating (SDS) electrophoresis, only two EBM isoforms with molecular masses of 38 kD and 37.5 kD were observed. Nonogaki and Morohashi (1999) attempted to immunblot lettuce EBM using polyclonal antibodies against one of the tomato EBM proteins. They managed to detect one band corresponding to EBM on immunoblots of SDS gels but none on immunoblots of native gels. They explained this

discrepancy with the fact that the antibodies were prepared using SDS-denaturated polypeptides.

There is little information regarding the nucleotide and protein structure of lettuce endo-beta-mannanase. Only one nucleotide sequence, encoding a lettuce EBM gene, exons 1-5, can be found in Genbank (accession # AJ315978). This gene sequence (3014 bp) has very limited homology to known nucleotide sequences of other EBM genes (tomato, coffee, *Arabidopsis thaliana*, and rice). However, the protein encoded by the lettuce EBM gene has strong homology to EBMs of coffee (68%), tomato (62%), rice (57%), and carrot (54%). It appears that endo-beta-mannanase catalytic and binding sites are preserved among the plant species.

A significant amount of work has been dedicated to elucidating the control of endo-beta-mannanase production and action. First, Halmer and Bewley (1979) hypothesized that EBM action was under embryo control. They observed differences in the time of appearance of EBM activity in intact seeds versus isolated endosperms. Additionally, in germinated seeds, the endosperm was completely degraded 25 hours from the start of imbibition. In contrast, isolated endosperms were visibly intact after 47 hours of imbibition, with only around 30% of their dry weight lost. Most attention was given to the EBM-ABA interaction. Halmer and Bewley (1979) were the first to report that isolated lettuce endosperms produced EBM only when incubated in a large volume of buffer (2 ml) but not in a small volume (0.2 ml). However, isolated endosperms were capable of producing EBM in a small volume if they had previously been incubated for at least 2 hours in a large buffer volume. Halmer and Bewley (1979) hypothesized that the endosperms were leaching inhibitor(s) in the buffer. This hypothesis was supported by

the observation that the buffer in which the endosperms had been rinsed was able to prevent EBM production in other endosperms. Dulson et al. (1988) identified the leached inhibitor as ABA. These authors reported that a leachate from lettuce endosperms was able to induce synthesis of ABA-specific proteins in barley aleuronic cells. Also, fractionation of endosperm leachate by either PVP-chromatography or HPLC resulted in coelution of ABA and the leachate component. Endosperms incubated in the presence of ABA at concentrations higher than 1 μM did not produce EBM and the inhibitory effect of ABA could not be overcome by application of different plant hormones (GA, BA, IAA, ethylene, or combinations of those) (Bewley and Halmer, 1980/81). However, control of EBM production in intact seeds appears to be more complicated than just depending on decreasing ABA. Dulson et al. (1988) reported that ABA did not decrease during germination. Bewley and Halmer (1980/81) reported that lettuce seeds with removed axes did not produce EBM. However, the axes could be substituted by the presence of BA or GA in the incubation solution but only if the seeds had intact cotyledons. This suggests that cotyledons play a part in the regulation of EBM activity. Halmer and Bewley (1979) hypothesized that the action of endo-β-mannanase is under the control of more factors than plant hormones. It appears that EBM production is under negative feedback regulation by products of endosperm degradation.

More information about a possible positive regulation of endo-beta-mannanase activity came from the work of Nascimento et al. (2000, 2001). This group correlated ethylene production with EBM activity and germination in several lettuce genotypes under different experimental conditions. Nascimento et al. (2000) reported that overall ethylene production was higher during the first 24 hours of imbibition in seeds of two

thermotolerant genotypes 'Everglades' and 'PI 251245' as compared to that in thermosensitive ' Dark Green Boston' and 'Valmaine' seeds at both 20ºC and 35ºC. 'Everglades' seeds produced more ethylene and more EBM during imbibition at 27.5ºC than at 20ºC or 35ºC. Also, artificially aged 'Everglades' seeds produced less ethylene during imbibition and germination at both 20ºC and 35ºC. The amount of ethylene produced correlated inversely with the duration of the accelerated aging. Nascimento et al. (2004a) observed that application of ACC, which significantly improved germination of 'Dark Green Boston' seeds at 35ºC, and increased ethylene production in 'Dark Green Boston' and 'Everglades' seeds , significantly enhanced EBM activity of both 'Dark Green Boston' and 'Everglades' at 35ºC. Nontreated 'Everglades' seeds produced 1.1 pmol/min EBM while nontreated 'Dark Green Boston' seeds did not produce EBM. In comparison, 'Dark Green Boston' treated with 10 mM ACC seeds produced 1.0 pmol/min enzyme prior to germination and ACC-treated 'Everglades' seeds produced 1.7 pmol/min. Imbibition in 20 mM STS decreased both 'Dark Green Boston' and 'Everglades' germination and delayed and decreased ethylene production. Simultaneously, STS inhibited EBM production in seeds from both genotypes. Inclusion of STS in a priming solution negated the promotive effect of priming on both germination and EBM production. Based on these observations, Nascimento et al. (2004a) hypothesized that ethylene participated in regulation of EBM activity. Ethylene may control transcription of the lettuce EBM gene(s). Leubner-Metzger et al. (1998) reported that ethylene stimulated production and action of β-1, 3-glucanase, an enzyme participating in endosperm weakening in tobacco seeds, and described an ethylene-responsive element (ERE) in its promoter. It is also possible that in lettuce ethylene

stimulates EBM indirectly through counteracting the effect of ABA. Antagonistic effects of ABA and ethylene on seed dormancy and germination have been reported in *Arabidopsis thaliana* (Beaudoin et al., 2000; Ghassemian et al., 2000).

In conclusion, there are some discrepancies concerning the role of endo-beta-mannanase in lettuce germination due to use of different experimental techniques and different cultivars. It appears that endo-beta-mannanase participates in germination of at least some lettuce genotypes (thermotolerant) under specific germination conditions (supraoptimal temperature).

Role of Endo-Beta-Mannanase in Other Plant Species

Endo-beta-mannanase has been reported to be a prerequisite for germination in several other plant species, including tomato, carrot, pepper, and white spruce. Nonogaki et al. (1992) observed development of EBM activity in tomato seeds following priming. Nomaguchi et al. (1995) reported development of galactomannan-hydrolyzing activity in micropylar endosperm of tomato seeds prior to germination. These authors observed activity only in the micropylar area in two different tomato cultivars. The strength of activity correlated with germination rate and final germination percentage. Using both native and denaturing protein electrophoretic techniques, Nonogaki and Morohashi (1996) observed one germination-specific and three post-germinative EBM isoforms in tomato seeds. The four EBM isoforms had different molecular weights, 39 kD for the germination-specific isoform as compared to 38 kD and 37.5 kD for the post-germinative proteins. The germination-specific and the post-germinative EBMs also had distinct temporal and spatial expressions. The germination-specific isoform was expressed only

in the micropylar region of tomato endosperm prior to germination while the post-germinative isoforms were expressed only in lateral part of the endosperm. The same group also reported that the products of galactomannan hydrolysis by these enzymes were different, with mannotetrose and mannopentose being predominant products for the germination-specific isoform. In 2000, Nonogaki et al. identified a germination-specific tomato EBM gene expressed only in micropylar endosperm prior to germination. The 1482 bp long cDNA contained an open reading frame encoding a mature protein of 393 amino acids plus a 22 amino acid long signal peptide. The overall amino acid sequence homology between the germination-specific and the post-germinative proteins was 78% and both the potential catalytic site and the potential glycosylation site were present in all isoforms. The predicted molecular weight of the germination-specific EBM was 44 kD with a pI of 5.7. Nonogaki et al. (2000) reported that GA-deficient tomato seeds (*gib-1*) did not geminate in water and no EBM production was detectable. If the *gib-1* seeds were imbibed in 100 μM GA, germination, EBM expression, and EBM activity were observed. Previously, Nomaguchi et al. (1995) observed that application of far-red light inhibited both germination and galactomannan-hydrolyzing activity (attributed later to EBM) in the micropylar endosperm. This inhibition was completely reversed upon application of red light. It appears that in tomato, EBM is under the control of a GA and light system.

Controversy has existed about the role of ABA in controlling EBM. Nomaguchi et al. (1995) reported that application of 10 μM ABA inhibited both germination and galactomannan-hydrolyzing activity in tomato seeds. However, Toorop et al. (1999) reported that the pattern of endo-beta-mannanase activity in tomato micropylar endosperm was not influenced by ABA and its structural analogues. Regardless of the

imbibition solution, the highest endo-beta-mannanase activity was observed between 50 and 100 hours of imbibition. Later, Nonogaki et al. (2000) did not observe any effect of ABA up to 100 μM concentration on both germination-specific EBM expression and activity. Interestingly, ABA suppressed activity of endo-beta-mannanase in the lateral tomato endosperm. This suggests that the germination-specific and post-germinative endo-beta-mannanase genes in tomato may have at least partially different control mechanisms. Toorop et al. (1999) confirmed that application of ABA was able to reduce lettuce EBM activity. It appears that EBM is only one of the factors regulating tomato germination.

Another plant species, in which endo-beta-mannanase is important for seed development and germination, is carrot (*Daucus carrota*). Homrichhausen et al. (2003) reported that carrot embryos did not complete their development prior to desiccation and apparently mature carrot seeds contained immature embryos that would finish development during seed imbibition. Homrichhausen et al. (2003) observed enlargement of the seed embryo during imbibition, paralleled with increase in size of the corrosion cavity surrounding the embryo. That was due to EBM activity. This group identified a 1317-bp long carrot EBM cDNA encoding a 387 amino acid mature protein. This protein has 59% amino acid identity with the germination-specific tomato EBM. Both the expression of this gene and the activity of the enzyme were observed in both micropylar and lateral regions of carrot endosperm. However, the activity in the micropylar region developed much earlier (18 hours) than the activity in the lateral region (24 hours). Radicle protrusion started at 36 hours after the start of imbibition. Application of ABA inhibited carrot germination but did not affect EBM.

Germination of several other plant species has been accompanied by an increase of galactomannan-hydrolyzing activity. Sanchez and de Miguel (1997) and later, Sanchez et al. (2002) reported that radicle protrusion in *Datura ferox* seeds was proceeded by weakening of the micropylar part of the endosperm , a decrease in its mannan content and an increase in the extractable activity of beta-mannanase. All these processes were promoted by application of red-light and inhibited by far-red light. Watkins et al. (1985) observed breakdown of the endosperm opposite the radicle tip in pepper seeds. This breakdown was accompanied by an increase in both seed cell wall degradating activity and galactomannan–hydrolyzing activity of extracts prepared from pepper seeds. Application of GA enhanced both degradating activities. Since galactomannan-hydrolyzing activity was observed, it was possible that endo-beta-mannanase was also present in pepper seeds. Since the completion of the *Arabidopsis thaliana* genome project, 5 *Arabidopsis* genes has been identified that have sequence homology to known EBM genes. However, there are no reports of their function in *Arabidopsis thaliana*.

Endo-beta-mannanase activity has also been associated with germination in gymnosperms. Downie et al. (1997) reported that the puncture force required to penetrate the structures surrounding a white spruce embryo (especially the megagamethophyte and the nucellus) decreased prior to radicle protrusion. Since both structures contained some mannan polymers, it was hypothesized that EBM might participate in their degradation. Indeed, a mannanase peak was observed in both dormant and nondormant white spruce seeds one day prior to radicle protrusion, being stronger in nondormant seeds.

Endo-beta-mannanase has been reported to participate in cell wall weakening during physiological processes other than seed germination. Dirk et al. (1995) reported

several EBM isozymes in different vegetative parts of alfalfa. The greatest number was observed in shoots, followed by roots and leaves. Carrington et al. (2002) identified an EBM gene that was expressed during ripening of tomato fruits. The identified 1426-bp cDNA encoded a 395 amino acid protein with a molecular weight of 42.4 kD. The pI of the enzyme was 8.82, while the pIs of seed-specific EBM proteins were between 4 and 5. The authors suggested that the amino acid composition of this enzyme was different from that of the seed-specific tomato EBMs. This hypothesis was supported by the low identity level between the fruit-specific and seed-specific proteins. The fruit-specific tomato EBM gene was first expressed in mature-green tomato fruits. The expression increased strongly at breaker stage and remained high until red and overripe stages. Interestingly, expression of this EBM gene was observed also in tomato flowers. The observance of EBM expression in tomato flowers and fruits together with the report of Bourgault and Bewley (2002) that the EBM protein was not detected in Never Ripe tomato mutant fruits led to the hypothesis that ethylene might participate in regulating EBM activity in tomato.

In summary, endo-beta-mannanase has a definite role in seed development and germination in certain plant species, including lettuce, tomato, carrot, *Daucus carrota,* and white spruce. However, establishing the exact importance of EBM for radicle protrusion is difficult since even in species like tomato, where a germination-specific EBM gene has been identified, germination that precedes EBM activity or inhibition of germination without inhibition of EBM has been observed. It is possible that EBM is required only during certain phases of germination (tomato) or under certain stressful conditions (lettuce).

Other Hydrolytic Enzymes with Possible Involvement in Lettuce Endosperm Weakening

Based on the structure of lettuce endosperm, two other enzymes have been hypothesized to participate in the endosperm weakening in lettuce.

α-Galactosidase

α-Galactosidase (EC 3.2.1.22), an enzyme responsible for removal of the β-1,6-galactose side chains from the galactomannan backbone, has been also hypothesized to be involved in endosperm weakening prior to germination in several plant species, including lettuce. In lettuce, Leung et al. (1979) reported α-galactosidase activity in both the cotyledons and the endosperm with that in the endosperm being higher. In the cotyledons, α-galactosidase was involved in mobilization of galactose-containing low molecular weight oligosaccharides (e.g. raffinose), while in the endosperm it was involved in degradation of galactomannan.

The hypothesis that α-galactosidase was involved in lettuce germination was proposed by Leung and Bewley (1981). They observed that this was the only enzyme whose activity increased in response to both red-light and GA prior to radicle protrusion. The strength of the α-galactosidase response was the same for red-light and GA. However, the response to red-light was slower and required prior imbibition of the seeds for at least 1.5 hours. Leung and Bewley (1981) also observed no correlation between α-galactosidase activity and germination under variety of germination conditions. Applications of both far-red light and ABA inhibited germination but did not affect α-galactosidase activity. The promotive effect of red-light and GA was in presence of already strong basal α-galactosidase activity. Later, Leung and Bewley (1981) reported

that α-galactosidase was able to release galactose only from products of prior endo-beta-mannanase activity which, most probably, meant that the action of α-galactosidase was not a prerequisite for EBM action, and consequently, for endosperm weakening.

The importance of α-galactosidase for radicle protrusion has been also extensively studied in tomato. Similar to lettuce, relatively high basal α-galactosidase activity was observed in tomato seeds. This activity slightly increased upon application of GA (Groot et al., 1988). Hilhorst et al. (1996) also reported that the activity of this enzyme was higher in endosperms of ABA-deficient mutants than wild-type seeds. Feurtado et al. (2001) observed α-galactosidase activity in micropylar and lateral regions of the endosperm and in embryo of tomato seeds, with the highest being that in the lateral endosperm. The activity remained constant throughout germination and post-germinative mobilization. The same group identified a 1540-bp α-galactosidase cDNA that encoded a 409 amino acid protein with a molecular weight of 44.9 kD and pI of 5.27. The protein had a signal peptide predicted to target it to the protein bodies. Mature tomato α-galactosidase protein had relatively high similarities with coffee and *Arabidopsis thaliana* α-galactosidases but there were much less similarities among the signal peptides of the proteins from the different species. This suggests that the enzyme in different plant species has different cellular localization. Neither the activity, nor the expression of tomato α-galactosidase was influenced by lower water potential or ABA. Based on the discrepancies between tomato germination and α-galactosidase activity, Feurtado et al. (2001) concluded that α-galactosidase did not participate in tomato germination. However, it seems that α-galactosidase action may be a prerequisite for germination in some plant species. McCleary and Matheson (1975) reported that the action of this

enzyme was required prior to action of endo-beta-mannanase and β-mannosidase in lucerne and guar.

B-Mannosidase

β-Mannosidase (exo-β-mannase, EC 3.2.1.25) has been also hypothesized to be involved in the process of endosperm weakening and germination. In lettuce seeds, Leung et al. (1979) detected β-mannosidase only in cotyledons. This group hypothesized that β-mannosidase was involved in degradation of oligomannan fragments, released from the endosperm to the cotyledons. Later, Quellette and Bewley (1986) reported that β-mannosidase activity was exclusively associated with cell walls of cotyledons and that it was constant during and following germination. Based on these observations, no role for β-mannosidase in endosperm weakening prior to germination was hypothesized.

The role of β-mannosidase in germination of tomato seeds has also been investigated. Mo and Bewley (2002) observed β-mannosidase activity during and following germination of tomato seeds and identified both a cDNA and a genomic clone for it. Contrary to tomato EBM activity and expression, the activity and expression of β-mannosidase were detected in both micropylar and lateral regions of the endosperm and in the embryo. Similar to EBM, β-mannosidase activity increased prior to radicle protrusion. The activity of this enzyme was lower in the micropylar region of nongerminated seeds than in germinated seeds, meaning that the ability for germination paralleled an increase in the activity of the enzyme. However, β-mannosidase activity increased prior to radicle protrusion also in the lateral endosperm. The authors identified a β-mannosidase cDNA encoding a 491 amino acid protein with molecular weight of 56.7

kD and pI of 6.8. Based on the observation of β-mannosidase activity prior to germination, Mo and Bewley (2002) concluded that this enzyme might be, together with EBM, one of the factors regulating radicle protrusion in tomato. It is possible that β-mannosidase participates indirectly in tomato germination through affecting the activity of endo-beta-mannanase. Sanchez et al. (2002) reported that low water potential inhibited activity of β-mannosidase and endosperm weakening in *Datura ferox* seeds. It is possible that decreased activity of β-mannosidase leads to accumulation of oligomannan fragments that may inhibit EBM activity. The possibility of inhibition of EBM activity by mannan oligosaccharides has been hypothesized for lettuce.

In summary, of the three enzymes participating in degradation of storage material in lettuce, based on their activity and on the work with other plant species, endo-beta-mannanase is probably the most important for endosperm weakening and radicle protrusion. However, there may be more factors than activity of this enzyme and osmotic potential of the lettuce embryo regulating germination. Nabors and Lang (1971) reported that weakening of endosperm cell walls might be mediated by an increase of cell wall extensibility. Chen et al. (2000) observed up-regulation of expression of a tomato expansin gene prior to tomato germination. Since lettuce endosperm contains other monosaccharides in addition to mannose and galactose, it is possible that other, yet unidentified enzymes, also participate in the endosperm weakening. One such potential candidate is L-arabinofuranosidase, an enzyme present in germinating barley grains and some ripening fruits (Grant and Briggs, 2002; Itai et al., 2003).

In conclusion, lettuce germination is complex, a net result of physiological processes occurring in the seed embryo and in the tissues enclosing it and under the

control of a number of factors, including light, maturation temperatures, hormones, and genetic background. Ethylene and endo-beta-mannanase appear to be two factors that strongly influence lettuce germination, at least under stressful conditions. Extending the work with ethylene action inhibitors to include lettuce genotypes with different genetic ability for germination and matured at different temperatures will allow better understanding of the role of ethylene in germination. Such an understanding will benefit from use of transgenic lettuce plants with altered ethylene perception or production.

CHAPTER 3
ETHYLENE ACTION INHIBITORS, SILVER THIOSULFATE (STS), AND 5-CHLORO-3-METHYL-4-NITRO-1H-PYRAZOLE (CMN-P) REDUCE GERMINATION OF THERMOSENSITIVE 'DARK GREEN BOSTON' AND THERMOTOLERANT 'EVERGLADES' SEEDS

Introduction

Ethylene has been implicated to play a role in germination of several different plant species, especially under stressful conditions. The role of ethylene in germination has been studied through the use of different inhibitors of ethylene production or action. Ethylene biosynthesis inhibitors, aminoethoxyvinylglycine (AVG), an inhibitor of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, and cobalt chloride, an inhibitor of ACC oxidase, have been predominantly used. Results from experiments with these inhibitors sometimes have been difficult to interpret due to the inhibitors' ability to influence other than ethylene-related biochemical processes. AVG, for example, is a general inhibitor of pyridoxal-phosphate-mediated enzyme reactions. Ethylene action inhibitors, such as 2,5-norbornadiene (NBD) and silver thiosulfate (STS), compounds that bind to ethylene receptors, have also been used to elucidate the role of ethylene in germination. These compounds compete with ethylene for receptor sites. After binding to the receptors, they prevent initiation of the ethylene-mediated signal transduction cascade. These compounds target ethylene action and, as such, shut down the effects of ethylene. Unfortunately, they can be difficult to use for research purposes.

Both NBD and trans-cyclooctane (TCO) have a strong unpleasant odor while diazocyclopentadiene is explosive.

The use of the ethylene inhibitors has contributed to a controversy surrounding the role of ethylene in germination. For example, Abeles (1986) reported that application of 1 mM AVG reduced ethylene production by approximately 50% in 'Grand Rapids' lettuce seeds at both optimal (25°C) and supraoptimal (30°C) temperatures. Simultaneously, AVG reduced 'Grand Rapids' germination in half at both temperatures. Saini et al. (1989) further reported that application of 1 mM AVG reduced both ethylene production and germination of 'Grand Rapids' seeds at 32°C in continuous red light. In both cases, the effect of AVG was reversed by ethylene. Nascimento et al. (2004a) observed complete inhibition of ethylene production by 10 mM AVG but there was no inhibition of germination in both 'Dark Green Boston' (DGB) and 'Everglades' (EVE) seeds at both 20°C and 35°C in light. However, when AVG was included in a priming solution, both the final percent germination and the germination rate of DGB and EVE seeds were reduced as compared to seeds primed in PEG only.

The use of ethylene action inhibitors like NBD has also led to controversial results. Abeles (1986) reported that application of increasing concentrations of NBD reduced 'Grand Rapids' germination at both 25°C and 30°C. At 30°C, the decrease in germination began at a lower concentration of NBD (1000 µl/l) than that at 25°C. Siriwitayawan et al. (2003) reported that NBD application reduced germination of 'Ailsa Craig' and 'Rutgers' tomato seeds 10 to 20%. The application of NBD significantly increased the time to reach 50% germination in both wild-type tomato seeds and ethylene insensitive seeds. Locke et al. (2000) reported that NBD application significantly reduced

barley germination. However, applications of both AVG and cobalt ions were unable to inhibit barley germination at 20°C in 16/8 hrs light/dark even though they inhibited ethylene production. These authors explained their results with a hypothesis that the inhibition of ethylene production could lead to channeling of S-adenosylmethionine (precursor of ACC and as such, of ethylene) towards polyamine synthesis, which, in turn, could stimulate germination. It is possible that the effect of ethylene biosynthesis or action inhibitors on germination in different plant species is related not only to the amount of ethylene produced and the extent to which ethylene is required for germination in these species, but also to the effect of altered ethylene biosynthesis or perception on other germination-regulating factors.

STS is one of the most extensively used ethylene action inhibitors. This compound has a unique mode of action: the silver ions displace copper ions, which are part of ethylene-binding receptors (Rodriguez et al., 1999). After the replacement of copper ions by silver ions, ethylene is still able to bind to the receptor but it is unable to initiate the downstream cascade of events leading to ethylene-mediated responses (Rodriguez et al., 1999). It is possible that the presence of silver ions instead of copper ions alters the duration of the binding of ethylene to the receptor. Sisler and Serek (2003) hypothesized that when a compound (ethylene action inhibitor) is bound to the receptor for a longer time than ethylene normally is, then the initiation of the downstream signal transduction cascade is disrupted.

STS affects germination in several plant species, including lettuce and tomato. Abeles (1986) reported that application of 1 mM STS slightly reduced 'Grand Rapids' germination at optimal temperature (25°C) but reduced it significantly at supraoptimal

temperature (30ºC). The effect of STS was negated if ethylene was applied simultaneously. Nascimento et al. (2004a) reported that application of 20 mM STS significantly reduced germination of thermosensitive DGB seeds at both 20ºC and 35ºC and of thermotolerant EVE seeds at 35ºC in light. The same author also observed delayed ethylene production following STS application. Similar to NBD, application of 2 mM STS increased the time to 50% germination in both wild-type and Never Ripe (NR) tomato seeds but did not affect their final germination (Siriwitayawan et al., 2003). In a similar way to the other ethylene inhibitors, the ability of STS to affect germination of different plant species also depends on their requirement for ethylene during germination.

Since all known ethylene action inhibitors have side effects, there is a continuous need for better, less toxic inhibitors. As a result of such a search, a compound called 5-chloro-3-methyl-4-nitro-pyrazole (CMN-P; known as 'Release') was discovered. CMN-P has been tested as an abscission agent in citrus. Application of CMN-P has induced abscission of mature 'Valencia' orange fruits but not of immature fruits, flowers, or leaves (Yuan et al., 2001). This compound can induce both ethylene production and ethylene-mediated gene expression (e.g. phenylalanine ammonia lyase) in mature 'Valencia' fruits, but not in other plant tissues (Yuan et al., 2001; Kostenyuk et al., 2002). However, application of CMN-P has also been reported to reduce perception of ethylene in *Arabidopsis thaliana* seedlings (Burns, personal communication).

Despite the existence of numerous reports describing a reduction in lettuce germination after application of STS, because of the use of different lettuce genotypes, STS concentrations and germination conditions, the relation between STS and germination remains unclear. The first objective of the present work was to examine the

effect of STS on two lettuce genotypes with documented thermotolerant (EVE) or thermosensitive (DGB) character. Comparison of the effect of STS on two genotypes with well-documented differences in germination ability at supraoptimal temperatures would provide valuable information about the extent to which sensitivity to ethylene is required by different lettuce genotypes under different germination conditions. The second objective was to establish whether CMN-P functions as an ethylene action inhibitor through its effect on lettuce germination and response to exogenous ACC.

Materials and Methods

Plant Material

Two lettuce cultivars, 'Everglades' (EVE), thermotolerant genotype, and 'Dark Green Boston' (DGB); thermosensitive genotype, were used. Thermotolerance is defined as the ability of seeds to germinate above 90% at temperatures up to 35°C in light (Nascimento, 1998). The seeds were produced in a commercial lettuce seed production field in 2000 in the San Joaquin Valley of California. The seeds were after-ripened for a year prior to use and stored at 10°C and 50% relative humidity.

Chemicals

Silver thiosulfate (STS) was prepared according to the procedure of Abeles (1986). Silver nitrate ($AgNO_3$) (Sigma, St. Louis, MO, USA) was mixed with sodium thiosulfate ($Na_2S_2O_3.5H_2O$) (Sigma, St. Louis, MO, USA) in a ratio of 1 ml silver nitrate to 4 ml sodium thiosulfate. Silver nitrate was added to sodium thiosulfate drop by drop, at high stirring speed in order to avoid precipitation. For the different experiments, STS

concentrations of 1, 5, 10, 20, and 50 mM were used. The STS solutions were prepared fresh prior to each experiment and stored in an aluminum covered container to prevent light degradation.

The inhibitor, 5-chloro-3-methyl-4-nitro-1H-pyrazole (CMN-P), was prepared by directly dissolving the compound in ultra-pure deionized water (Millipore, Ontario, Canada). The solid powder was obtained from Dr. J. Burns (Univ. of Florida, Lake Alfred). For the different experiments, CMN-P concentrations of 0.1, 0.5, 1, 2, and 5 mM were used.

The ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), was prepared by directly dissolving the powder (Sigma, St. Louis, MO, USA) in ultra-pure water. For the different experiments, ACC concentrations of 10 and 20 mM were used. Both CMN-P and ACC solutions were prepared fresh prior to each experiment.

Germination Tests

For each germination experiment, three or four replications per treatment of 20 or 30 seeds were used. The seeds were placed on one layer of 5 cm in diameter Whatmann filter paper #1, moistened with 1 ml deionized, ultra-pure water. The filter paper was re-moistened as needed by adding 0.5 ml water. The blotters were covered with 5.5 cm Petri dish lids and incubated at 20°C or 35°C under constant light (30 μmol $m^{-2}s^{-1}$) in Precision Scientific (Winchester, VA, USA) incubators. Germination was recorded at radicle protrusion. The number of germinated seeds was counted at 10, 12, 16, 20, 24, 36, 48, 72, and 120 hours after the germination had been initiated. The percent germination at 120 hours was considered final percent germination.

The germination experiments with STS and CMN-P, during which a broad range of temperatures was tested, were conducted on a thermogradient table (Van Dok and de Boer db 5000 89-1; Enkhuizen, The Netherlands) under 20 μmol $m^{-2}s^{-1}$ constant light. The temperatures used for the STS experiments were: 20, 24, 28, 32, and 36°C, and for the CMN-P experiments: 14, 20, 24, 28, 32, 36, and 40°C.

Ethylene Sensitivity Assay

The sensitivity of DGB and EVE seeds to exogenously supplied ACC in presence of ethylene action inhibitors (STS or CMN-P) was examined using a triple response bioassay according to Bleecker et al. (1988). Three replications (40 seeds each) per treatment were placed on 1% Phytagar (GibcoBRL, Gaithersburg, MA, USA) medium supplemented with 0, 3, 10, or 100 μM ACC. The medium was sterilized by autoclaving for 25 min at 100°C/1.1 kg/cm^2. Afterward, 80 ml medium were dispensed per phytotray (Sigma, St. Louis, Mo, USA). The appropriate concentration of ACC was added after the medium was cooled. STS or CMN-P were added to final concentrations of 1 mM for STS and 0.1 and 0.5 mM for CMN-P to half of the medium. The seeds were sterilized with 20% commercial bleach solution for 20 min prior to placement on the medium, followed by rinsing with sterile deionized water three times. The seedlings were grown at 20°C in continuous darkness for 10 days. On the 10^{th} day, both the radicle and the hypocotyl length were measured for each seedling.

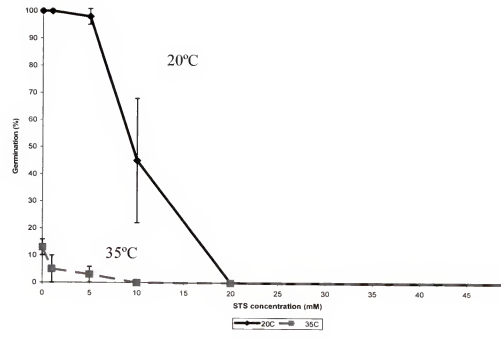
Experimental Design and Statistical Analysis

All experiments were conducted using a randomized complete block design, with temperature being the block. Data were analyzed using analysis of variance procedure (ANOVA) performed by means of Statistical System (SAS) software (SAS, 1999). Means were separated by Least Significant Difference (LSD) values. Significance levels of P=0.01 and P=0.05 were used.

Results and Discussion

At 20°C, DGB seeds germinated at 100% in water and in 1 and 5 mM STS (Figure 3-1). At 20ºC, DGB germination was reduced in 10 mM STS when compared to the 0 mM STS and completely inhibited at STS concentrations equal to or higher than 20 mM. At 35ºC, in 0 mM STS, DGB germination was significantly lower than DGB germination at 20°C. Application of 5 mM or higher STS concentrations further reduced DGB germination at 35°C (Figure 3-1). At 20ºC, EVE germination was reduced by STS at a concentration of 50 mM. At 35ºC, however, application of 20 mM STS reduced EVE germination and 50 mM STS completely inhibited it (Figure 3-1).

Application of the same concentration of STS affected in different way thermosensitive DGB and thermotolerant EVE seeds. Differences in the effect of STS on lettuce germination in relation to genotype have been reported by other authors. Abeles (1986) reported that seeds from the thermosensitive and photosensitive cultivar 'Grand Rapids' had reduced germination in 1 mM STS at both 25ºC and 30ºC. Nascimento et al. (2000) observed that application of 20 mM STS significantly reduced DGB germination



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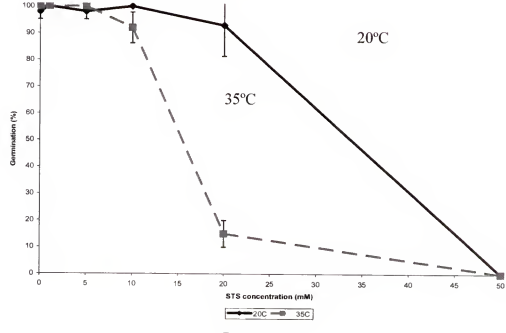

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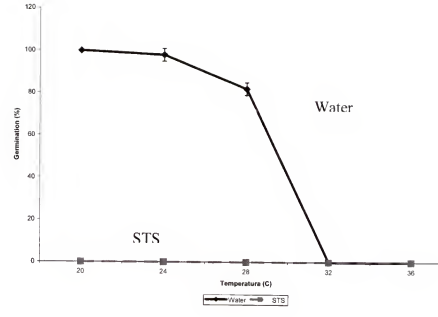
Figure 3-1. Germination in light at 20ºC and 35ºC, in increasing STS concentrations. Data were analyzed using ANOVA. Means for the tow genotypes for the three way interaction, genotype X temperature X solution were separated using Least Significant Difference (LSD) with value of 9.3. A) DGB. B) EVE.

at 20ºC. Application of STS more strongly affects seeds that have higher sensitivity to temperature during germination. It is possible that seeds from a thermosensitive genotype require perception of more ethylene in order to germinate. Seeds from thermotolerant genotypes produce more ethylene during germination than thermosensitive seeds. Nascimento et al., (2000) reported that EVE seeds produced approximately 10 times more ethylene when germinated at both 20ºC and 35ºC than DGB seeds. Prusinski and Khan (1990) reported that there was a strong positive correlation between the amount of ethylene produced and germination of seeds from nine lettuce cultivars under stressful conditions. Ethylene production from slit seeds of these nine cultivars and germination at 32°C and 35°C had correlation coefficients of 0.80 indicating that ethylene was the main factor determining the ability of these seeds to germinate under high temperatures. There may be a relation between the amount of ethylene produced and the ability of STS to affect germination of DGB and EVE seeds.

Since it was observed that STS differentially affected DGB and EVE germination at optimal (20°C) and supraoptimal (35°C) temperatures, it was decided to examine the effect of 20 mM STS on DGB and EVE germination at a range of temperatures (from 20° to 36°C) (Figure 3-2). With no STS added, DGB germination was reduced at 28ºC and completely inhibited at 32ºC and 36ºC. In 20 mM STS, DGB seeds did not germinate at any temperature. EVE germination in water was not affected by increases in temperature from 20ºC to 36ºC (Figure 3-2). In 20 mM STS, EVE germination was reduced at temperatures above 28°C.

The increase in STS-mediated inhibition of germination at increasing temperatures can be explained by increased requirements for ethylene during germination



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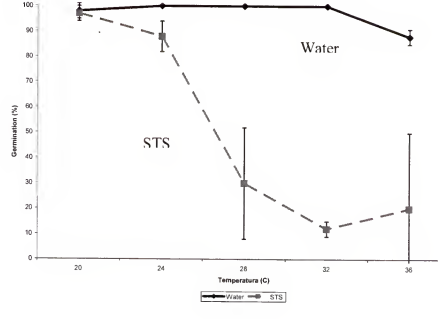

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Figure 3-2. Germination in light in water and 20 mM STS, at increasing temperatures (from 20 to 36°C). Data were analyzed using ANOVA. Means for the two genotypes for three way interaction, genotype X temperature X solution were separated using Least Significant Difference (LSD) with value of 14.1. A) DGB. B) EVE.

at supraoptimal temperatures. A stronger STS effect at high temperatures has been reported by other authors (Abeles, 1986; Nascimento et al., 2004a). It is possible that in order for the lettuce seeds to germinate at high temperature, they have to produce and perceive more ethylene.

The reason for the possible higher requirement for ethylene during germination at supraoptimal temperature is unknown. Nascimento et al. (2000) correlated higher ethylene production with higher activity of the enzyme endo-beta-mannanase during lettuce germination at supraoptimal temperature. The authors hypothesized that higher activity of the enzyme might be required for lettuce to germinate under supraoptimal temperature. Prusinski and Khan (1990) reported that at high temperature lettuce seeds from nine different cultivars had lower germination potential than at optimal temperature. Seeds with the greatest germination potential also produced the greatest amount of ethylene and had better germination than the low ethylene producing cultivars.

The pattern of germination of EVE seeds in 20 mM STS resembled that of DGB seeds in water (Figure 3-2). This indicated that EVE seeds could be sensitized to temperature when ethylene perception was reduced.

It is possible that STS-mediated germination inhibition is due to the effect of STS on other processes with importance for germination and unrelated to ethylene. In order to test this possibility, the effect of STS on germination in the presence of ACC was examined (Table 3-1). At 20ºC, DGB seeds germinated at 100% in water and 10 mM ACC. DGB germination was strongly inhibited at both temperatures in 20 mM STS. The inhibition was almost completely reversed by simultaneous application of ACC. At 35ºC, DGB seeds did not germinate unless ACC was added. In contrast to DGB, EVE

Table 3-1 Germination in light of DGB and EVE in water, 20 mM STS, 10 mM ACC and combination of STS and ACC, at 20ºC and 35ºC

Solution	Cultivar/Temperature			
	DGB		EVE	
	20ºC	35ºC	20ºC	35ºC
Water Control	100.0	5.0	100.0	100.0
20 mM STS	5.0	0.0	98.3	35.0
10 mM ACC	100.0	91.7	100.0	98.3
20 mM STS + 10 mM ACC	80.0	1.7	96.7	90.0
LSD Value		13.2		

Data were analyzed using ANOVA. Means were separated using Least Significant Difference (LSD) for the three way interaction, solution, temperature and genotype.

germination was similar in all solutions at 20°C (Table 3-1). At 35°C, EVE germination was only reduced in 20 mM STS.

The hypothesis that ethylene is required for germination is supported by the increase in germination following ACC application (Table 3-1). Application of ACC has been reported to increase the speed of germination of both wild-type and ethylene insensitive 'Rutgers' and 'Ailsa Craig' tomato seeds (Siriwitayawan et al., 2003). Khan (1994b) reported that application of ACC stimulated both ethylene production and germination in aged seeds of cabbage, tomato, snap bean and sweet corn.

During our experiments, lettuce seeds germinated faster in ACC than in water (Figure 3-3). At 20°C, DGB seeds completed germination in water at 36 hours as compared to 24 hours in 10 mM ACC. At 35°C, EVE seeds reached more than 90% germination at 16 hours in water as compared to 10 hours in 10 mM ACC. There was no difference in the speed of germination of EVE and DGB seeds in water and in 10 mM ACC at 20°C.

It is possible that when ACC is provided to the seeds it reduces the need for *in vivo* ACC synthesis. Further, the 10 mM ACC concentration given may far exceed that which the seed produces naturally, which, in turn, leads to strongly increased ethylene production and earlier germination. The ineffectiveness of ACC on germination speed of EVE seeds at 20°C may indicate that under these conditions EVE seeds produce a sufficient amount of ethylene to ensure optimal germination.

The ability of DGB seeds to respond to exogenously supplied ACC indicates that DGB seeds probably do not differ from EVE seeds in their ability to convert ACC to ethylene and that the difference in their ethylene producing capacity may lay in the steps

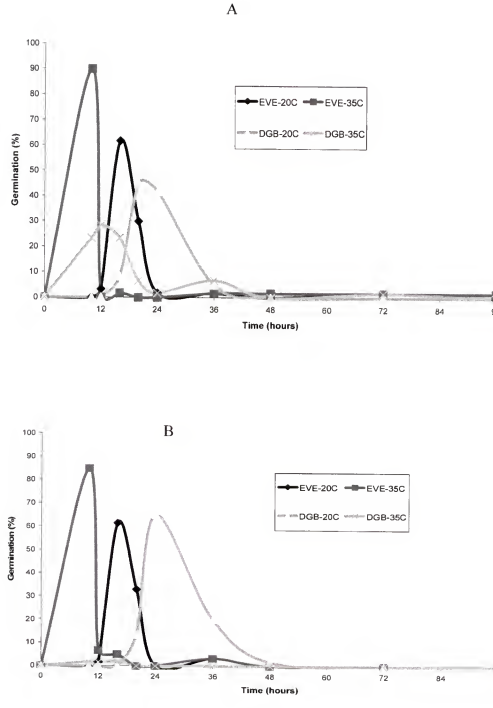


Figure 3-3 Speed of germination of DGB and EVE seeds in water and 10 mM ACC over 4 days imbibition period at 20°C and 35°C in continuous light. A) Water. B) 10 mM ACC.

leading to ACC synthesis. This also indicates that the conversion of ACC to ethylene can proceed uninhibited at supraoptimal temperature, at least in EVE and DGB genotypes.

The observation that application of ACC can reverse the effect of STS indicates that this effect is due to inhibition of ethylene action and not due to general cellular toxicity. The exact mechanism of ACC-mediated reversal is unknown. Since lettuce seeds germinate in ACC faster than in water or STS, it is possible that ethylene is produced and binds to the receptors faster than STS can tie up the sites thus inhibiting germination.

Lettuce germination appears to potentially be a dynamic process with respect to ethylene production and perception. The inhibition of both DGB (at 20°C) and EVE (at 20° and 35°C) germination by STS can be reversed if the seeds are transferred to water. Twelve hours after transfer from 20 mM STS to water, 90% of the EVE seeds germinated. By 48 hours, EVE seed germination was 100%. The phenotype of EVE seedlings imbibed initially in STS and later transferred to water suggested long term effect of STS on lettuce seedlings (Figure 3-4). It can be speculated that the reversal of STS-mediated germination inhibition may be due to synthesis of new receptors, which, in absence of a continuous supply of STS, will bind ethylene and initiate the signal cascade leading to germination.

In order to obtain more evidence for the need and potential role of ethylene in germination at high temperature, another potential ethylene action inhibitor, 5-chloro-3-methyl-4-nitro-1H-pyrazole (CMN-P) was used. The same types of experiments were performed with CMN-P as with STS with the exception that only EVE seeds were used.

Figure 3-4 Comparison of phenotype of imbibed at 35°C in water for 5 days or in 20 mM STS for one day, followed by imbibition in water for 4 days EVE seedlings. The initially imbibed in 20 mM STS EVE seedlings had longer and thinner roots indicating possible lower root sensitivity to ethylene that that of imbibed only in water seedlings. A) Water only. B) STS followed by water.

EVE seeds germinated at almost 100% at both 20°C and 35°C in water (Table 3-2). Germination at 20°C was not affected by increasing CMN-P concentrations until 5 mM CMN-P, at which EVE germination was completely inhibited. At 35°C, EVE germination was significantly reduced in 0.5 mM CMN-P (Table 3-2). Imbibition in 1 mM CMN-P reduced germination even further, to 36%, and a concentration of 5 mM completely inhibited germination.

The similarity between the response of EVE seeds to CMN-P and STS suggested that CMN-P might possibly function as an ethylene action inhibitor. The physiologically active CMN-P concentrations appeared to be approximately 10 times lower than that of STS suggesting possible stronger sensitivity to CMN-P than STS.

The effect of two CMN-P concentrations (0.5 mM and 1 mM) on EVE germination at a range of temperatures (from 14° to 40°C) was examined (Table 3-3). The purpose of this experiment was to examine whether CMN-P, like STS, would effect EVE germination more strongly at supraoptimal temperature than at optimal temperature. In water, EVE germination was significantly reduced (85%) at 36°C and almost completely inhibited (11%) at 40°C. When EVE seeds were imbibed in 0.5 mM CMN-P, their germination was reduced at 32°C and completely inhibited at 40°C (Table 3-3). In 1 mM CMN-P, EVE seeds germinated significantly less at 28°C than at 24°C. At 32°C, their germination was further reduced and almost completely inhibited at 36°C and 40°C (Table 3-3).

The data presented in Table 3-3 provide further evidence for the action of CMN-P as an ethylene inhibitor. Application of 0.5 mM CMN-P increased thermosensitivity of EVE similar to that observed for STS.

Table 3-2 EVE germination in light at 20°C and 35°C in water and increasing CMN-P concentrations

CMN-P Concentration (mM)	Temperature	
	20°C	35°C
Water Control	100.0	95.6
0.1	100.0	97.8
0.5	96.7	64.5
1	96.7	35.6
5	0.0	0.0
LSD Value	12.1	

Data were analyzed using ANOVA. Means were separated using Least Significant Difference (LSD) for the two way interaction, CMN-P and temperature.

Table 3-3 EVE germination in light in water, 0.5 mM CMN-P, and 1 mM CMN-P at increasing temperatures

Temperature (°C)	Solution		
	EVE		
	Water	0.5 mM CMN-P	1 mM CMN-P
14°C	100.0	98.8	88.8
20°C	100.0	96.3	90.0
24°C	100.0	97.5	88.8
28°C	98.8	91.3	66.3
32°C	97.5	65.0	16.7
36°C	85.0	20.0	5.0
40°C	11.3	0.0	0.0
	LSD Value 11.3		

Data were analyzed using ANOVA. Means were separated using Least Significant Difference (LSD) for the two way interaction, temperature and solution.

Increasing the CMN-P concentration to 1 mM led to a further decrease in the temperature range at which EVE germination was thermoinhibited. The temperature at which the first reduction effect of CMN-P on germination was observed was 28°C. This is the same temperature at which both DGB germination in water and EVE germination in 20 mM STS began decreasing (Figure 3-2).

The hypothesis that CMN-P affects lettuce germination by decreasing perception to ethylene was examined using simultaneous application of ACC and CMN-P (Table 3-4). At 20°C, EVE seeds had nearly 100% germination in all solutions except for the combination of 1 mM CMN-P and 10 mM ACC, in which they germinated at 78%. At 35°C, EVE germination was significantly reduced in both 0.5 mM CMN-P and 1 mM CMN-P, more strongly in 1 mM CMN-P. Simultaneous application of 10 mM ACC and CMN-P increased significantly EVE germination in 0.5 mM CMN-P, from 56% to 66%. Still, EVE germination in the combination of the two solutions was significantly reduced in comparison to that in water or ACC alone (Table 3-4). Application of ACC to 1 mM CMN-P did not improve EVE germination. Since EVE seeds are thermotolerant, application of ACC alone did not affect their final germination at 20°C or at 35°C. Application of ACC simultaneously with CMN-P increased EVE germination above the levels of that in CMN-P alone although the increase was lower than for STS.

Fewer EVE seeds germinated at 35°C following transfer from CMN-P to water as compared to the STS to water transfer. The percent germination for EVE seeds imbibed at 35°C for the first 24 hours in 0.5 mM CMN-P, followed by imbibition for 96 hours in water was 69% as compared to 56% for 0.5 mM imbibed seeds only. If EVE seeds were transferred from 1 mM CMN-P, the percent germinated seeds was 34% as compared to

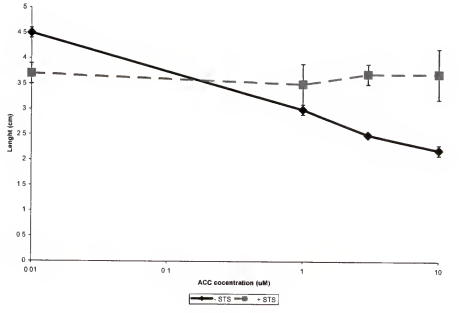
Table 3-4 Germination in light of EVE at 20ºC and 35ºC in water, 0.5mM CMN-P, 1mM CMN-P, 10 mM ACC and combinations of 10 mM ACC and CMN-P

Solution	Temperature	
	20ºC	35ºC
Water Control	100.0	100.0
0.5mM CMN-P	96.7	55.5
1mM CMN-P	94.4	27.8
10mM ACC	98.9	100.0
0.5 mM CMN-P + 10 mM ACC	97.8	65.9
1 mM CMN-P + 10 mM ACC	77.8	33.3
LSD Value	9.9	

Data were analyzed using ANOVA. Means were separated using Least Significant Difference (LSD) for the two way interaction, solution and temperature.

21% for 1 mM CMN-P imbibed seeds. The difference between the full reversion of the STS-mediated inhibition by ACC and the partial reversion of CMN-P-mediated inhibition might be explained by the hypothesis that lower CMN-P than STS concentrations might be required to inhibit ethylene perception. The reason for the suspected difference between the effective CMN-P and STS concentrations is unknown. Based on the chemical structure of CMN-P, which resembles that of known cyclic olefin ethylene inhibitors, it might be speculated that if this compound acts as an ethylene inhibitor, it would be through competition with ethylene for binding to the receptor. This is a different mechanism from that hypothesized for STS. EVE seeds germinated at 100% when they were transferred from CMN-P to water and imbibed at 20ºC. This suggested that the CMN-P effect on EVE germination was not due to general cellular toxicity and that the process that was affected by CMN-P was only required for EVE germination at supraoptimal temperature.

In order to test whether the different responses of DGB and EVE seeds to STS were related to different sensitivity to ethylene, a triple response test was employed. The ability of different ethylene action inhibitors (STS, NBD, and TCO) to null the triple response has been previously reported (Hirayama et al., 1999; Madlung et al., 1999; Nicolas et al., 2001). It is possible that the different lettuce genotypes differ in their sensitivity to ethylene, and, consequently, to inhibition of ethylene synthesis or perception. Abeles (1986) observed reduction of 'Grand Rapids' germination at 25ºC by 1 mM STS while Nascimento et al. (2004a) reported that EVE germination at 20ºC was not affected by 20 mM STS.



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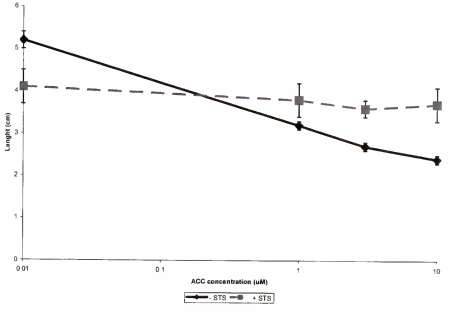
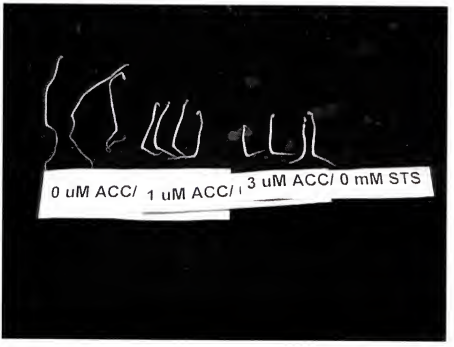


B

Figure 3-5 Decrease of hypocotyl length (cm) of EVE and DGB seedlings on increasing ACC concentrations. The ACC concentrations are represented in a lg scale. Vertical bars represent standard errors. A) EVE. B) DGB.

The length of DGB and EVE hypocotyls in the presence of increasing ACC concentrations and STS is presented in Figure 3-5. In the absence of STS, the average hypocotyl length for DGB and EVE hypocotyls decreased significantly when 1 μM ACC was added to the medium (Figure 3-5). There was further decrease in DGB and EVE hypocotyl length when the ACC concentration was increased to 3 μM. When 1 mM STS was present in the medium, there was no significant difference between the hypocotyl length of DGB or EVE seedlings in either the absence or presence of ACC, regardless of the ACC concentration. Both DGB and EVE hypocotyls were longer in presence of STS than in absence of STS for all ACC concentrations (Figure 3-5).

Exposure of seedlings to exogenous ethylene or ACC results in the development of a distinct seedling phenotype, called 'triple response'. This phenotype is characterized by an exaggeration of apical curvature, thickening and shortening of the hypocotyls and shortening of the radicles (Bleecker et al., 1988). Simultaneous application of STS and ACC resulted in a reversal of the 'triple response' phenotype and development of a phenotype observed in ethylene insensitive seedlings (Figure 3-6). When STS was present in the medium, EVE seedlings did not develop a 'hook' and in some cases the cotyledons were open. Both the hypocotyl and the radicle were thinner and the radicles did not have root hairs. Similar counteraction of the 'triple response' phenotype by STS was observed in other plant species (Nicolas et al., 2001). The phenotype of EVE seedlings grown during the triple response bioassay was similar to that of EVE seedlings exposed to STS during imbibition. This indicates that STS, most probably, affects EVE seeds during germination in the same way as EVE seedlings during triple response,



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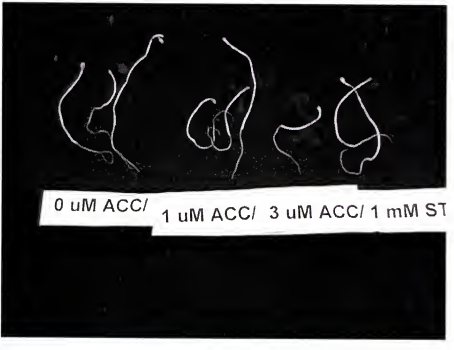


B

Figure 3-6 Comparison of phenotype of EVE seedlings grown on increasing ACC concentrations for 10 days at 20˚C in dark in absence and presence of 1 mM STS. On increasing ACC concentrations, in absence of STS, EVE seedlings exhibited typical 'triple response' phenotype. None of the phenotypic characteristics of the 'triple response' was observed in presence of 1 mM STS. A) No STS. B) 1 mM STS.

through reduction of ethylene perception. The triple response bioassay indicated that there was no difference in sensitivity to ethylene between DGB and EVE seedlings. In order to determine whether CMN-P might be an inhibitor for ethylene receptor sites in lettuce, a CMN-P triple response was done for EVE (Figure 3-7). In the absence of CMN-P, EVE hypocotyls were significantly shorter in the presence of 0.3 μM ACC as compared to 0 ACC. Increase of the ACC concentration to 1 or 3 μM led to a further significant decrease in EVE hypocotyl length. EVE hypocotyls grown in presence of 100 μM CMN-P were significantly shorter than EVE hypocotyls, grown in absence of CMN-P, regardless of the ACC concentration (Figure 3-7).

The hypocotyls of seedlings grown in the presence of CMN-P were thicker than those of EVE seedlings grown in absence of CMN-P regardless of ACC concentration. In this respect, the EVE phenotype resembled that of seedlings overproducing ethylene (*eto*) or having a constitutively induced triple response (*ctr*) (Figure 3-8). However, there were some differences. The average radicle length of seedlings grown on CMN-P plus ACC was similar to that of seedlings grown on ACC alone and the radicles had few if any root hairs. There was also no 'hook' formation in seedlings grown on CMN-P and, in some cases, the cotyledons were open.

The triple response bioassay did not confirm that CMN-P could reduce perception of ethylene in a similar way to STS. Additionally, CMN-P appears to increase either the sensitivity to ethylene or the production of ethylene during the triple response bioassay. Since CMN-P had been reported to induce ethylene production in the abscission zone of mature 'Valencia' oranges (Yuan et al., 2001), it was more probable that CMN-P stimulated ethylene production rather than perception.

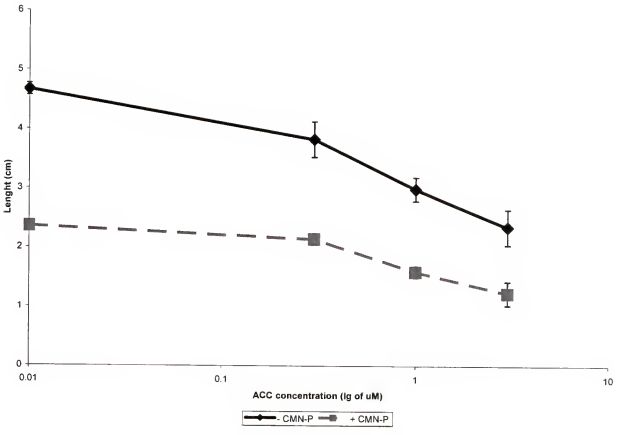
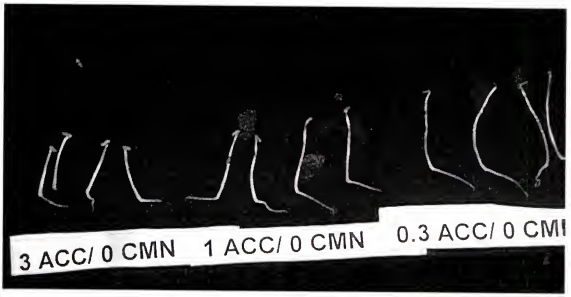


Figure 3-7 Decrease in hypocotyl length of EVE seedlings grown in continuous dark for 10 days at 20°C in presence and absence of 0.1 mM CMN-P. ACC concentrations are represented as lg of μM. Vertical bars represent standard errors.



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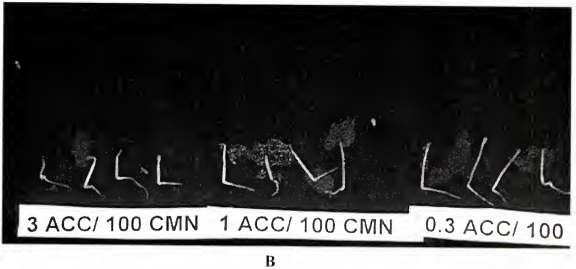


B

Figure 3-8 Phenotype of EVE seedlings grown at 20°C for 10 days on the following ACC concentrations (0, 0.3, 1 and 3 μM) in presence (100 μM) and absence of CMN-P. Regardless of ACC concentration, EVE seedlings grown in presence of CMN-P had shorter hypocotyls than seedlings grown in absence of CMN-P. In presence of CMN-P EVE seedlings did not form apical hook. A) No CMN-P. B) 100 μM CMN-P.

However, it cannot be excluded that CMN-P inhibits hypocotyl growth during the triple response through some unrelated to ethylene processes.

The reason for the different effect of CMN-P on EVE germination and EVE triple response is unknown. The mechanism of action of CMN-P is also unknown. It may be speculated that CMN-P affects differently separate ethylene-mediated physiological processes. CMN-P is considered to promote only abscission of mature orange fruits but not that of immature fruits or leaves (Yuan et al., 2001). During the triple response bioassay, CMN-P induced shortening of the hypocotyls, but not formation of an apical hook or root hair growth.

Uncoupling of the different phenotypic affects of the triple response has been observed in a class of *Arabidopsis* mutants (hookless, *hls*; Guzman and Ecker, 1990). It may be speculated that the different effect of CMN-P on different ethylene-mediated physiological processes may be due to prevalence of different ethylene receptors or compounds involved in the ethylene signal transduction during these processes.

In conclusion, CMN-P appears to affect EVE germination in the same concentration- and temperature-dependent manner as the ethylene action inhibitor, STS. However, the effect of CMN-P on the morphology of EVE seedlings grown in presence of exogenous ACC (triple response) supports the hypothesis that CMN-P acts as a stimulator of ethylene production, unlike STS. In order to conclude whether CMN-P affects EVE germination through inhibition of ethylene action or inhibition of other processes preceding germination, more experiments with the compound are necessary. It will be useful to observe whether CMN-P can affect germination of other lettuce cultivars like DGB or other plant species. It will also be interesting to observe the effect of CMN-P

on other ethylene-dependent physiological processes like flowering in order to observe whether, during any of these processes, CMN-P inhibits ethylene perception.

Summary

Application of the ethylene action inhibitor, silver thiosulfate (STS) reduced germination of the thermotolerant lettuce genotype 'Everglades' (EVE) at supraoptimal temperature and germination of the thermosensitive genotype 'Dark Green Boston' (DGB) at optimal and supraoptimal temperatures. DGB germination was affected at lower STS concentrations than EVE germination, indicating that DGB seeds are more sensitive to STS, probably, due to lower ethylene availability. DGB germination was affected by STS at lower temperatures than EVE germination, possibly due to the ability of EVE seeds to produce more ethylene than DGB seeds at high temperatures. Nascimento et al. (2000) reported that EVE seeds produced approximately 10 times more ethylene than DGB seeds at both optimal and supraoptimal temperatures in light. The STS effect on both genotypes was totally reversed by simultaneous application of ACC, indicating that the STS effect was ethylene-specific. DGB and EVE seedlings demonstrated similar sensitivity to exogenous ACC and STS during triple response experiments, which suggested that the differences in the STS effect on seeds from these two genotypes were due to differences in ethylene availability. The abscission initiating compound, 5-chloro-3-methyl-4-nitro-1H-pyrazole (CMN-P) inhibited EVE germination in a similar concentration- and temperature-dependent manner to that of STS. However, the phenotype of EVE seedlings grown in presence of both ACC and CMN-P during the triple response experiments did not support the hypothesis that this compound acted as an

ethylene action inhibitor. The reason for the discrepancy between the effects of CMN-P on these two different physiological processes is unclear. It may be speculated that the ability of CMN-P to act as an ethylene promoter or inhibitor depends either on the type of the physiological process or that CMN-P has pleiotropic effects, only partially related to the ethylene effect on lettuce seeds and seedlings.

CHAPTER 4
MATURATION OF SEEDS FROM THERMOSENSITIVE AND THERMOTOLERANT LETTUCE GENOTYPES AT ELEVATED TEMPERATURES RESULTS IN INCREASED ETHYLENE PRODUCTION AND GERMINATION AT SUPRAOPTIMAL TEMPERATURE

Introduction

The ability of lettuce to germinate at supraoptimal temperatures (higher than 28°C) depends on both the genotype and the environmental conditions during seed development and maturation. Many lettuce cultivars (e.g. 'Grand Rapids' and 'Pacific') are both thermosensitive and photosensitive. Gray et al. (1975) and Thompson et al. (1979) attempted to correlate thermotolerance with physical characteristics of the lettuce seed like testa color, but no definite correlation was made.

Germination of certain thermotolerant and thermosensitive lettuce cultivars can be improved by seed maturation at elevated temperatures. Sung (1996) reported that germination of the thermosensitive 'Dark Green Boston' (DGB) genotype and the thermotolerant 'Everglades' (EVE), 'PI 251245', and 'Floricos 83' genotypes, matured at 30°/20°C or 35°/25°C was greater than that of seeds matured at 25°/15°C or 20°/10°C at a range of supraoptimal temperatures (27°, 30°, 33°, and 36°C). Even though germination of 30°/20°C matured seeds was higher at all supraoptimal temperatures, it still decreased with an increase in temperature. Maturation at 30°/20°C and 35°/25°C also led to a decrease in the mean time to germination in both thermotolerant and thermosensitive seeds. Sung (1996) observed that seeds of some cultivars like 'Valmaine' did not have

improved germination following maturation at 30°/20°C or 35°/25°C. The ability of the high temperature exposure to affect germination depends on lettuce genotype.

Several authors have tried to identify the mechanism(s) of the promotive effect of supraoptimal environmental temperatures on germination. Weakening of the micropylar endosperm is considered to be a prerequisite for lettuce germination, especially under supraoptimal temperature (Nascimento et al., 2000; Cantliffe et al., 2000). Using puncture force measurements, Sung et al. (1998) observed that the resistance of the micropylar endosperm was lower for both DGB and EVE seeds matured at 30°/20°C than in seeds matured at 20°/10°C. The same authors also reported that both DGB and EVE seeds matured at 20°/10°C had more endosperm cells in front of the radicle than seeds matured at 30°/20°C. It is possible that the thicker endosperm layer of seeds matured at 20°/10°C physically more strongly restricts their germination than does the thinner endosperm of seeds matured at 30°/20°C. Jones (1974) and later Sung (1996) hypothesized that the endosperm weakening was mediated by enzymes, produced in the endosperm cells. Nascimento et al. (2000) observed activity of the enzyme endo-beta-mannanase (EBM) in both DGB and EVE seeds matured at either 30°/20°C and 20°/10°C prior to germination. The endosperm cells of lettuce are rich in galactomannans, a key substrate for this enzyme. Thus, degradation of the cell wall of the endosperm cells weakens the endosperm allowing the radicle to push through it. Nascimento et al. (2000) reported that the activity of EBM was significantly higher in seeds matured at 30°/20°C as compared to seeds matured at 20°/10°C, during incubation at both 20°C and 35°C. It appears that the improved germination of seeds matured at 30°/20°C as compared to

20°/10°C seeds may be the result of greater EBM activity, leading to endosperm weakening.

Ethylene also has been correlated with lettuce germination, especially at supraoptimal temperature. Nascimento et al. (2000) reported that thermotolerant lettuce genotypes produced significantly more ethylene during germination than thermosensitive genotypes. Artificial seed aging decreased both the production of ethylene and the ability for germination at supraoptimal temperature (Nascimento et al., 2004b). Prusinski and Khan (1990) reported that ethylene production from nine different genotypes of lettuce correlated with their ability to germinate at both 32°C and 35°C as well as under salt and osmotic stresses. Abeles (1986) observed full reversion of thermoinhibition of 'Grand Rapids' seeds at 30°C in dark by 10 µl/l ethylene.

Since the amount of ethylene produced correlates with the ability of lettuce to germinate at supraoptimal temperature and application of exogenous ethylene stimulates lettuce germination under thermoinhibitory conditions, it can be hypothesized that ethylene plays an important role in lettuce germination at high temperature. The objective of the present work was to examine whether thermosensitive and thermotolerant lettuce seeds matured at 30°/20°C produced more ethylene or had higher sensitivity to ethylene than seeds produced at 20°/10°C, which resulted in higher germination of the 30°/20°C seeds.

Materials and Methods

Plant Material

Two lettuce (*Lactuca sativa* L.) genotypes, 'Everglades' (EVE), a thermotolerant genotype, and 'Dark Green Boston' (DGB), a thermosensitive genotype, were used. Thermotolerance was defined as the ability of seeds to germinate above 90% at temperatures up to 35°C in light (Nascimento, 1998). All seeds were produced in the spring of 1999 at the University of Florida in Conviron E15 (Controlled Environments Inc., Pembina, ND, USA) growth chambers. The seeds were produced under a photoperiod of 12 hours light /12 hours dark and two different day/night temperature regimes, 30°/20°C or 20°/10°C. At maturity, which was determined visually by the extent of drying of the flowers and seed color development (usually 3 weeks post pollination), the seeds were harvested and cleaned manually. They were stored at 10°C and 50% relative humidity until use.

Germination Tests

Three replications of 20 seeds per treatment were placed on one layer Whatmann #1 filter paper, moistened with 1 ml deionized, ultra-pure water (Millipore, Ontario, Canada). The filter paper was re-moistened as needed by adding 0.5 ml water. The blotters were covered with 5.5 cm Petri dish lids. Germination was conducted at either 20°C or 36°C at either constant light (30 $\mu mol\ m^{-2}s^{-1}$) or constant dark in Precision Scientific (Winchester, VA, USA) incubators. Dark germination was counted under a green safe light. A temperature of 36°C was designated supraoptimal instead of 35°C

because 36°C allows better distinction between seed lots with high and low ability for germination at supraoptimal temperature (Cantliffe, personal communication). Radicle protrusion was recorded as germination. The number of germinated seeds was counted at 10, 12, 16, 20, 24, 36, 48, 72, and 120 hours after imbibition was initiated. The percent germination at 120 hours was considered the final percent germination.

In order to examine the effect of reduced ethylene perception or increased ethylene production on germination of DGB and EVE seeds matured at either 20°/10°C or 30°/20°C, their germination in the ethylene action inhibitor, silver thiosulfate (STS), or the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), or a combination of both was tested. The different solutions were prepared as described in chapter 3.

Ethylene Determination

Seeds from each seed lot (30) were placed on one layer of 6 cm long by 0.5 cm wide Whatmann #2 filter paper inside a plastic 1.2 X 7.5 cm culture tube (Fisher Scientific Co., Fairlawn, NJ, USA). The tubes were kept completely sealed using rubber stoppers (Fisher Scientific Co.) and were immobilized in a horizontal position to the bottom of transparent seed germination trays using tape. The paper strip was moistened with 0.3 ml water at the start of the experiment and 0.15 ml water was added as necessary. The culture tubes were placed at 20°C or 36°C in either continuous light (30 $\mu mol\ m^{-2}s^{-1}$) or continuous dark. Ethylene was measured at 9, 12, 15, 18, 21, 24, 36, and 48 hours after the imbibition was initiated. Germination was determined immediately prior to ethylene measurement. A 1 ml gas sample was withdrawn using a gas-tight hypodermic syringe (Fisher Scientific Co.). The tubes were flushed with air and re-sealed

for the next sampling. Each treatment was replicated three times. Ethylene was assayed using a gas chromatograph (Hewlett Packard Series II 5890) equipped with a flame ionization detector. The carrier gas was nitrogen, the oven, injector and detector temperatures were 130°C, 110°C, and 150°C, respectively. The conditions of the gas chromatography are described in detail by O'Donnell et al. (2003).

Ethylene Sensitivity Assay

The sensitivity of both DGB and EVE seeds matured at either 20°/10°C or 30°/20°C to exogenously supplied ACC was measured using a triple response bioassay according to Bleecker et al. (1988). Three replications (25 seeds each) per treatment were placed on 1% Phytagar (GibcoBRL, Gaithersburg, MA, USA) medium supplemented with 0, 3, 10, or 100 μM ACC. The medium was sterilized by autoclaving for 25 min at 100°C/1.1 kg/cm^2 pressure, and 80 ml were dispensed per phytotray (Sigma, St. Louis, MO, USA). The appropriate concentration of ACC was added after the medium had cooled. Seeds were sterilized with 20% commercial bleach solution (Publix, Lakeland, FL, USA) for 20 minutes followed by rinsing with sterile water (three times) prior to placement on the medium. The seedlings were grown at 20°C in continuous dark for 10 days. On the 10th day, both radicle and hypocotyl length were measured for each seedling.

Experimental Design and Statistical Analysis

All experiments were conducted with three replications per treatment using randomized complete block design, with temperature being the block. The data were

analyzed using analysis of variance procedure (ANOVA) performed by means of Statistical System (SAS) software (SAS, 1999). Means were separated by Least Significant Difference (LSD) test. The significance levels used were P=0.01 and P=0.05.

Results and Discussion

Germination of both DGB and EVE seeds, matured under either temperature conditions, 20°/10°C or 30°/20°C, was 100% at 20°C in both constant light and dark (Table 4-1, Table 4-2), indicating good seed viability. In light, at 20°C, all seed lots germinated less in 20 mM STS than in water, with the exception of EVE 30°/20°C seeds. DGB 30°/20°C seeds had greater germination than DGB 20°/10°C in 20mM STS. In light, at 36°C, germination of seed lots matured at 20°/10°C was significantly lower than their germination at 20°C or germination of 30°/20°C seed lots (Table 4-1). When the seeds were imbibed at 36°C in STS, DGB and EVE seed lots matured at 20°/10°C did not germinate while seed lots matured at 30°/20°C germinated at 9% (DGB) and 22% (EVE).

DGB and EVE seeds, matured at 20°/10°C or 30°/20°C, had similar germination in dark as in light (Table 4-2). The main difference was that the percent germinated seeds was lower at all imbibition conditions than that in light with the exception of 20°C in water. Seed lots matured at 30°/20°C once again germinated significantly better than seeds matured at 20°/10°C (Table 4-2). When the seeds were imbibed at 36°C in 20 mM STS, they did not germinate.

The ability of certain lettuce genotypes to germinate at supraoptimal temperatures has been well documented (Sung et al., 1998; Nascimento et al., 2000). Germination of all lettuce genotypes can be enhanced by application of ethylene (Abeles, 1986). An

Table 4-1 Germination of DGB and EVE seeds, matured at 30˚/20˚C or 20˚/10˚C, at 20°C and 36°C in light, in water or 20 mM STS

	Temperature/Solution			
	20°C		36°C	
Seed Lot	H_2O	STS	H_2O	STS
DGB 20°/10	100.0	4.3	6.7	0.0
DGB 30°/20°	100.0	26.7	84.7	9.0
EVE 20°/10°	100.0	40.0	31.3	0.0
EVE 30°/20°	100.0	97.7	97.7	22.3
LSD value		11.8		

Data were analyzed using ANOVA. Means were separated using Least Significant Difference (LSD) for the three way interaction, STS, temperature and seed lot.

Table 4-2 Germination of DGB and EVE seeds, matured at 30°/20C or 20°/10C, at 20°C and 36°C in dark, in water or 20 mM STS

	Temperature/Solution			
	20°C		36°C	
Seed Lot	H_2O	STS	H_2O	STS
DGB 20°/10°	100.0	4.7	2.3	0.0
DGB 30°/20°	100.0	44.3	46.7	0.0
EVE 20°/10°	100.0	18.0	4.7	0.0
EVE 30°/20°	100.0	91.0	73.3	2.3
LSD Value		10.2		

Data were analyzed using ANOVA. Means were separated using Least Significant Difference (LSD) for the three way interaction, STS, temperature and seed lot.

ethylene action inhibitor, silver thiosulfate, has been shown to inhibit germination (Abeles, 1986; Nascimento et al., 2004a). Nascimento et al. (2004a) reported that while germination of both DGB and EVE was affected at 35°C by 20 mM STS only DGB germination was affected at 20°C. Kozarewa (Chapter 3) reported that EVE was less sensitive to STS at increasing temperatures as compared to DGB. Seed maturation at 30°/20°C as compared to maturation at 20°/10°C appears to decrease the sensitivity to STS, possibly either thorough an increase in sensitivity to ethylene or an increase in ethylene production. The sensitivity to STS, which indicated stronger dependence of germination on ethylene, was higher at 36°C than at 20°C. Prusinski and Khan (1990) and Nascimento et al. (2000) hypothesized that at supraoptimal temperature lettuce seeds had an increased requirement for ethylene or that possibly ethylene production was reduced. Prusinski and Khan (1990) based their hypothesis on the observation that lettuce seeds and seedlings were more sensitive to ethylene under stressful conditions (including high temperature) than under optimal conditions and that conversion of ACC to ethylene was reduced by an increase of the imbibition temperature from 25°C to 35°C. Nascimento et al. (2000) observed significantly higher ethylene production at both optimal and supraoptimal temperature in lettuce genotypes capable of germination at supraoptimal temperature. The inhibitory effect of STS was greater at 36°C in dark. It may be speculated that the reason for this was the combined stronger requirement for ethylene at the supraoptimal temperature and the possible decreased ethylene production in dark as compared to that in light. Such a decrease has been reported by several authors (Saini et al., 1989; Nascimento et al., 2000).

In order to determine if temperature affected ethylene production of seeds matured at 20°/10°C or 30°/20°C, ethylene was measured as seeds germinated at 20°C and 36°C in continuous light and dark (Tables 4-3 and 4-4). At 20°C, all seeds germinated at 100% or close to 100%, regardless of light or dark imbibition conditions. At 36°C, in light, all seeds germinated above 80%. Germination of EVE seeds matured at 30°/20°C was significantly higher than germination of EVE 20°/10°C seeds (Table 4-3). At 36°C, in dark, all seed lots with the exception of EVE 30/20°C had significantly reduced germination as compared to their germination at 36°C, in light. Seeds matured at 20°/10°C had strongly inhibited and lower germination than seeds matured at 30°/20°C (Table 4-3).

At 20°C, in light, seeds matured at 30°/20°C produced more ethylene than seeds matured at 20°/10°C. Imbibition in dark at 20°C reduced significantly ethylene production in all seeds as compared to that in light (Table 4-4). Both DGB and EVE 30°/20°C seeds did not produce more ethylene in dark at 20°C than did DGB and EVE 20°/10°C. Imbibition at 36°C in light significantly increased ethylene production in seeds matured at 30°/20°C as compared to their ethylene production at 20°C in light. In contrast, ethylene production in seeds matured at 20°/10°C was similar or lower than their production at 20°C in light (Table 4-4). Imbibition in dark, at 36°C, significantly reduced ethylene production in all seeds. However, the ethylene production by 30°/20°C seeds still remained higher than that from 20°/10°C seeds (Table 4-4).

Differences in ethylene production between seed lots matured at 30°/20°C and 20°/10°C might possibly account for differences in their ability to germinate. Under all imbibition conditions, except imbibition at 20°C in dark, seeds matured at 30°/20°C

Table 4-3 Germination of DGB and EVE seeds, matured at 20°/10°C or 30°/20°C, at 20°C or 36°C in light or dark

	20°C		36°C	
Seed Lot	Light	Dark	Light	Dark
DGB 20/10	97.8	95.6	90.0	10.0
DGB 30/20	98.9	98.9	96.7	82.2
EVE 20/10	98.9	98.9	82.2	15.6
EVE 30/20	98.9	100.0	100.0	98.9
LSD value		7.7		

The germination percentages were analyzed using ANOVA. The percent germination after 48 hours of imbibition was considered final and used for the analysis. Means were separated using Least Significant Difference (LSD) for the three way interaction temperature X seed lot X light or dark conditions.

Table 4-4 Ethylene production of DGB and EVE seeds, matured at 20°/10°C or 30°/20°C, at 20°C or 36°C in light or dark

	20°C		36°C	
Seed Lot	Light	Dark	Light	Dark
DGB 20/10	30.51	13.62	36.41	4.49
DGB 30/20	43.75	19.53	71.51	35.35
EVE 20/10	30.24	11.49	24.96	4.04
EVE 30/20	46.62	17.65	82.44	29.40
LSD value		7.9		

The average ethylene production was analyzed using ANOVA. Ethylene values were calculated averaging ethylene production at 9, 12, 15, 18, 21, 24, 36, and 48 hours of imbibition. Means were separated using Least Significant Difference (LSD) value for the three way interaction seed lot X temperature X light or dark.

produced more ethylene than seeds matured at 20°/10°C. The higher ethylene production by 30°/20°C seeds at 36°C, in both continuous light and continuous dark, possibly, resulted in higher germination of these seed lots than that of 20°/10°C seeds.

Imbibition in dark led to reduced ethylene production in all seed lots, regardless of the temperature of imbibition. Nascimento (1998) reported that EVE seeds produced less ethylene when germinated in dark compared to light at 20°C, 27.5°C or 35°C. Regardless of the decreased ethylene production, all seed lots germinated at 100% at 20°C (Table 4-4). This suggests that either ethylene is not required for germination at this temperature or that the ethylene produced is sufficient to promote germination. It has been hypothesized (Chapter 3) that the requirements for ethylene increase at supraoptimal temperature. This hypothesis is supported by the observation that germination of DGB and EVE 30°/20°C at 36°C in both light and dark is accompanied by higher ethylene production as compared to ethylene production at 20°C. Another evidence in support of this hypothesis is that seeds matured at 20°/10°C, which have similar ethylene production at 36°C in light to that at 20°C in light, have lower germination at 36°C as compared to that at 20°C. (Tables 4-3 and 4-4). It is possible that the reduction in ethylene production in dark results in inability of the seeds matured at 20°/10°C to meet the increased requirement for ethylene at the supraoptimal temperature, which ultimately results in their low germination. Nascimento et al. (2000) observed that seeds from thermosensitive lettuce genotypes had both lower germination and lower ethylene production at supraoptimal temperatures versus optimal ones. The reason for the inability of 20°/10°C seeds to meet the increased requirements for ethylene at supraoptimal temperatures is unknown. It may be hypothesized that 20°/10°C seeds are less capable either to

synthesize ACC or to convert ACC to ethylene at supraoptimal temperatures. Khan (1994b) reported that more vigorous lettuce seeds had greater ability to convert exogenously supplied ACC to ethylene and to germinate under stressful conditions than did seeds with low vigor. Siriwitayawan et al. (2003) also reported that tomato seeds with high vigor were able to convert ACC more efficiently under stressful conditions than low vigor tomato seeds.

In order to measure ethylene the tubes were sealed for periods of 3 hours. This led to a build up of ethylene, which led to greater germination of the seed lots at high temperature than that of seeds germinated in unsealed Petri dishes (compare Tables 4-1 and 4-2 and Table 4-3).

The time course of ethylene production during germination of DGB and EVE seed lots matured at 20°/10°C or 30°/20°C at 20°C or 36°C in light is represented in Figures 4-1 to 4-4. At 20°C, EVE 30°/20°C reached germination close to 100% earlier than did EVE 20°/10°C, at 15 hours versus 24 hours, respectively (Figures 4-1, 4-2). Simultaneously, the peak of ethylene production at 20°C was earlier for EVE 30°/20°C seeds than for EVE 20°/10°C, at 18 hours versus 21 hours. The peak amount of ethylene produced was greater for EVE 30°/20°C than for EVE 20°/10°C seeds. At 36°C, similar to 20°C, germination of EVE 30°/20°C seeds commenced earlier than germination of EVE 20°/10°C. EVE 30°/20°C reached close to 100% germination at 15 hours, while germination of EVE 20°/10°C reached 82% at 48 hours. The peak of ethylene production was also earlier for EVE 30°/20°C at 36°C than for EVE 20°/10°C.

At 20°C, DGB 30°/20°C completed germination faster than DGB 20°/10°C, at 18 hours versus 21 hours (Figures 4-3, 4-4). Both DGB seed lots produced highest amounts

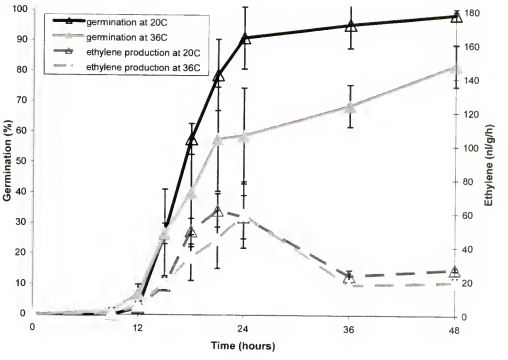


Figure 4-1. Germination (solid lines) and ethylene production (broken lines) of EVE 20°/10°C seeds imbibed at 20°C (open triangles) and 36°C (filled triangles) in light. Vertical bars indicate standard errors. The tubes were sealed for three hours between the measurements, with the exception of time points 36 and 48, when they were sealed for 12 hours.

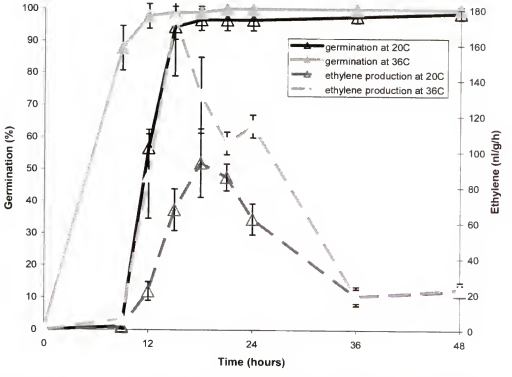


Figure 4-2. Germination (solid lines) and ethylene production (broken lines) of EVE 30°/20°C seeds imbibed at 20°C (open triangles) and 36°C (filled triangles) in light. Vertical bars indicate standard errors.

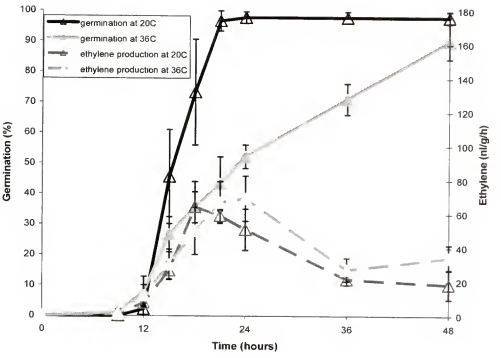


Figure 4-3. Germination (solid lines) and ethylene production (broken lines) of DGB 20°/10°C seeds imbibed at 20°C (open triangles) and 36°C (filled triangles) in light. Vertical bars indicate standard errors.

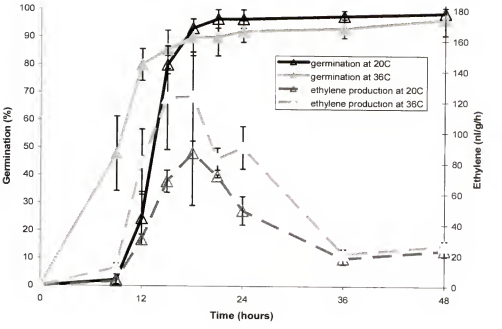


Figure 4-4. Germination (solid lines) and ethylene production (broken lines) of DGB 30°/20°C seeds imbibed at 20°C (open triangles) and 36°C (filled triangles) in light. Vertical bars indicate standard errors.

of ethylene at 18 hours. At 36°C, DGB 30°/20°C seeds reached 90% germination at 18 hours while DGB 20°/10°C reached 90% germination at 48 hours. DGB 30°/20°C seeds had the highest ethylene production at 15 and 18 hours after the start of the imbibition while ethylene production in DGB 20°/10°C peaked at 24 hours. DGB 30°/20°C seeds produced approximately two times more ethylene at peak than DGB 20°/10°C seeds (Figures 4-3, 4-4).

Ethylene evolution in all seed lots, at all temperatures, decreased after germination was completed. All seed lots had peak ethylene production either at the time period when germination was completed or at the time periods immediately preceding or following the time of germination completion. This indicates that ethylene production results from the germination process and is not just a consequence of seedling growth and development. Ethylene production varied among the three replications when the percent germinated seeds was also variable.

Similar to the imbibition in light, in dark, at 20°C, EVE 30°/20°C again germinated faster than EVE 20°/10°C. EVE 30°/20°C completed germination at 18 hours versus 21 hours for EVE 20°/10°C seeds (Figures 4-5, 4-6). Ethylene production of EVE 20°/10°C peaked at 21 hours while ethylene production of EVE 30°/20°C peaked at 24 hours. At 36°C, germination of EVE 20°/10°C was much lower than germination of EVE 30°/20°C, 19% versus 98%. EVE 30°/20°C seeds reached close to 100% germination at 24 hours when they also had highest ethylene production. In dark, at 20°C, DGB 30°/20°C seeds completed germination much earlier than DGB 20°/10°C seeds, at 15 hours versus 24 hours (Figures 4-7, 4-8). The peak of ethylene production was at 24 hours for DGB 20°/10°C and in the interval between 15 to 24 hours for DGB 30°/20°C.

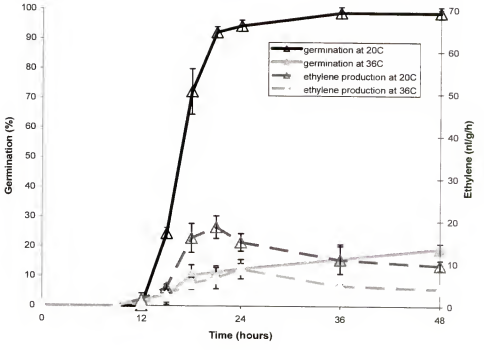


Figure 4-5. Germination (solid lines) and ethylene production (broken lines) of EVE 20°/10°C seeds imbibed at 20°C (open triangles) and 36°C (filled triangles) in dark. Vertical bars indicate standard errors. The tubes were sealed for three hours between the measurements, with the exception of time points 36 and 48, when they were sealed for 12 hours.

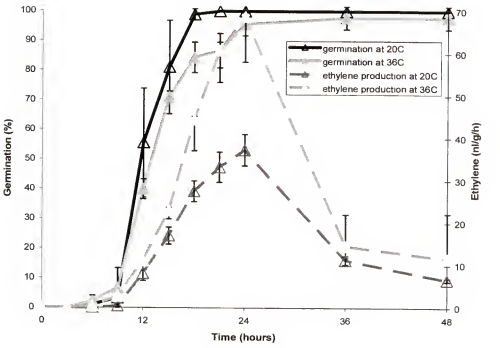


Figure 4-6. Germination (solid lines) and ethylene production (broken lines) of EVE 30°/20°C seeds imbibed at 20°C (open triangles) and 36°C (filled triangles) in dark. Vertical bars indicate standard errors.

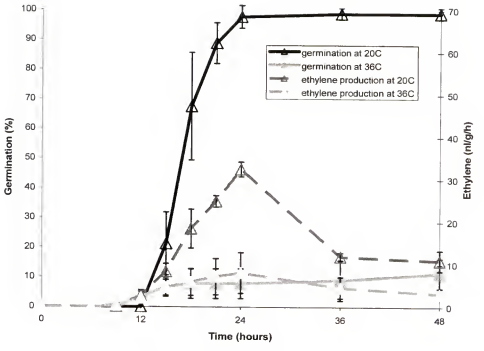


Figure 4-7. Germination (solid lines) and ethylene production (broken lines) of DGB 20°/10°C imbibed at 20°C (open triangles) and 36°C (filled triangles) in dark. Vertical bars indicate standard errors.

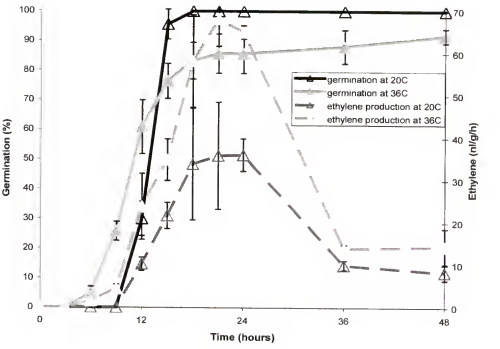


Figure 4-8. Germination (solid lines) and ethylene production (broken lines) of DGB 30°/20°C seeds imbibed at 20°C (open triangles) and 36°C (filled triangles) in dark. Vertical bars indicate standard errors.

DGB seeds matured at either temperature produced similar peak amounts of ethylene at 20°C. At 36°C, germination of DGB 20°/10°C seeds was much lower than germination of DGB 30°/20°C, 11% as compared to 92%. DGB 30°/20°C germination increased throughout the whole imbibition to reach 92% at 48 hours. DGB 30°/20°C produced most ethylene in the interval between 21 and 24 hours.

The ethylene determination during germination of DGB and EVE seeds matured at either 20°/10°C or 30°/20°C indicates that the ability of both DGB and EVE seeds matured at 30°/20°C to produce more ethylene leads to their higher germination at the supraoptimal temperature. Especially in dark, at supraoptimal temperatures, seeds matured at 20°/10°C were unable to produce enough ethylene to trigger their germination. The reason for the increased ability to produce ethylene by seeds matured at 30°/20°C is unknown. No studies have been directed towards understanding of the effect of different seed maturation conditions on germination either in lettuce or in other plant species. It may be possible that some of the biosynthetic steps leading to ethylene production are enhanced during seed maturation at 30°/20°C. The activities of ACC synthase, the enzyme converting S-adenosylmethionine to ACC, and ACC oxidase, the enzyme converting ACC to ethylene, would be the most probable targets. These two enzymes have been reported to be subject to complex regulation throughout plant development leading to strict regulation of ethylene production. A decrease in ACC oxidase activity during seed aging has been observed. Khan (1994b) reported that both naturally and artificially aged lettuce seeds that had lower ability to germinate under stressful conditions also had lower ability to convert ACC to ethylene. Siriwitayawan et al. (2003) also reported that low-vigor tomato and sweet corn seeds had lower abundance

of transcripts for ACC oxidase despite having similar amounts of ACC as the high-vigor seeds. It may be hypothesized that maturation at elevated temperatures undergoes a process that is opposite to that found during seed aging when the ability to produce ethylene and, consequently, to germinate is enhanced.

The responses of both DGB and EVE seeds matured at either 20°/10°C or 30°/20°C to exogenously supplied ACC and ACC/STS combinations were examined in order to determine whether seeds matured at the two different temperature conditions responded differently to the ethylene precursor or the inhibitor of ethylene perception. At 20°C, in light, both DGB and EVE seed lots matured at either 20°/10°C or 30°/20°C germinated at 100% in either water or 10 mM ACC (Table 4-5). When imbibition was conducted in 20 mM STS, germination of all seed lots was significantly reduced. When the seeds were germinated in combination of 10 mM ACC and 20 mM STS, their germination was significantly increased as compared to germination in STS alone. At 36°C in light, in water, germination of both DGB and EVE seed lots matured at 30°/20°C was significantly higher that germination of DGB and EVE 20°/10°C. Imbibition in 20 mM STS reduced strongly the percent germinated seeds in all seeds lots. Imbibition in 10mM ACC increased germination significantly.

In dark, all seeds germinated at 100% or close to 100% in either water or 10mM ACC at 20°C (Table 4-6). Imbibition in 20 mM STS significantly reduced germination of all seeds lots when compared to that in water or ACC. When ACC was added to STS, germination of all seed lots was significantly improved as compared to germination in STS alone (Table 4-6). At 36°C, in water, germination of all seed lots was reduced when compared to germination at 20°C. Application of 10 mM ACC significantly increased the

Table 4-5 Germination of DGB and EVE seeds matured at different temperatures in 10 mM ACC, 20 mM STS , and 10 mM ACC + 20 mM STS at 20°C and 36°C in light

Seed Lot	Temperature/Solution							
	20°C				36C			
	H_2O	STS	ACC	ACC + STS	H_2O	STS	ACC	ACC + STS
DGB 20/10	100.0	0.0	100.0	40.0	26.7	0.0	73.3	1.7
DGB 30/20	100.0	35.0	100.0	71.7	80.0	10.0	98.3	15.0
EVE 20/10	100.0	28.3	100.0	76.7	33.3	0.0	86.7	3.3
EVE 30/20	100.0	88.3	100.0	98.3	100.0	23.3	100.0	76.7
LSD value				10.4				

Data were analyzed using ANOVA. Means were separated using Least Significant Difference (LSD) for the three way interaction, STS, temperature and seed lot.

percent germinated seeds of all seed lots. The increase in germination was much greater for seed lots matured at 30°/20°C (Table 4-6) of all four seed lots. Imbibition in 20 mM STS inhibited germination of all seed lots at 36°C. Simultaneous application of ACC was able to reverse the STS effect only in EVE 30°/20°C seeds.

The speed and uniformity of germination of DGB and EVE seeds matured at 20°/10°C and 30°/20°C during germination at 20°C and 36°C in water, 10 mM ACC, 20 mM STS and a combination of ACC and STS in either continuous light or continuous dark is represented in Figures 4-9 and 4-10. In light, at 20°C, in water, the peak of germination was earlier for seeds matured at 30°/20°C than for seeds matured at 20°/10°C, at 12 and 16 hours as compared to 20 hours, respectively. At 36°C, in water, seeds matured at 20°/10°C had very low germination, while germination of 30°/20°C seeds was rapid and uniform, with peak of germination at 12 hours. At 20°C, in light, application of ACC did not increase the speed of germination of any seed lot. Application of ACC at 36°C in light also did not increase the speed of germination of any of the seed lots but increased the uniformity of their germination allowing almost all seeds matured at 30°/20°C to germinate at a single imbibition time point. When 20 mM STS was applied to the seeds at 20°C in light, their germination was delayed. At 36°C, germination of all seed lots was both delayed and reduced in STS. At 20°C, in a combination of STS and ACC, germination of all seeds was delayed as in STS alone but higher than in STS. When a combination of STS and ACC was applied to the seeds at 36°C, their germination was as rapid as that in water or in ACC.

Table 4-6 Germination of DGB and EVE seeds matured at different temperatures in 10 mM ACC, 20 mM STS and 10 mM ACC + 20 mM STS at 20°C and 36°C in dark

Seed Lot	Temperature/Solution							
	20°C				36°C			
	H_2O	STS	ACC	ACC + STS	H_2O	STS	ACC	ACC + STS
DGB 20/10	98.3	5.0	100.0	35.0	3.3	0.0	15.0	0.0
DGB 30/20	100.0	48.3	100.0	90.0	63.3	0.0	93.3	3.3
EVE 20/10	100.0	1.3	100.0	18.3	0.0	0.0	20.0	0.0
EVE 30/20	100.0	63.3	100.0	100.0	66.7	5.0	96.7	33.3
	LSD value				8.5			

Data were analyzed using ANOVA. Means were separated using Least Significant Difference (LSD) for the three way interaction, STS, temperature and seed lot.

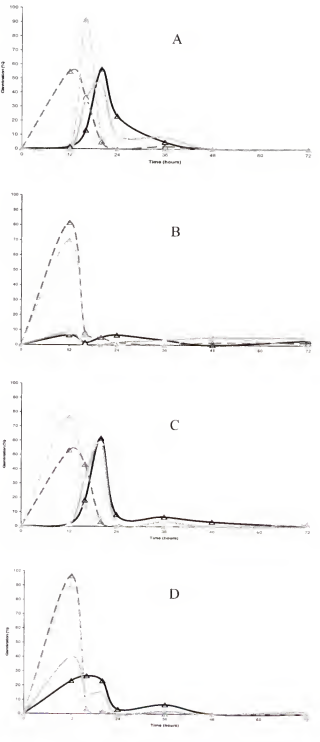


Figure 4-9. Uniformity of germination of DGB and EVE seeds matured at 20°/10°C or 30°/20°C during germination at 20°C or 36°C in continuous light. Seeds matured at 30°/20°C were represented with broken lines while those matured at 20°/10°C were represented with solid lines. Open triangles represent EVE and filled triangles represent DGB. A) 20°C/water; B) 36°C/water; C) 20°C/ACC; D) 36°C/ACC; E) 20°C/STS; F) 36°C/STS; G) 20°C/ ACC + STS; H) 36°C/ ACC + STS.

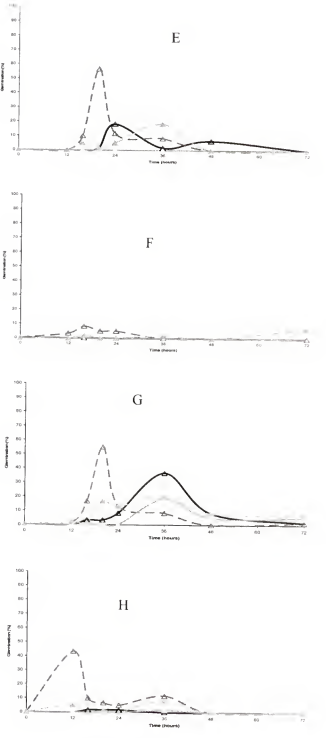


Figure 4-9. Continued.

In dark, at 20°C, in water, all seed lots had a peak of germination at 20 hours, meaning that germination of 30°/20°C seeds was delayed as compared to that in light (Figure 4-10). At 36°C, in dark, germination of seed lots matured at 20°/10°C was almost completely inhibited. Germination of 30°/20°C seeds was reduced with peak germination occurring at 12 hours. At both 20°C and 36°C, application of ACC did not increase the speed of germination but increased its uniformity (Figure 4-10). At 36°C, ACC did not affect germination of seeds matured at 20°/10°C. At 20°C, imbibition in 20 mM STS delayed the peak of germination in all seed lots, from 20 to 36 hours. At 36°C, in dark, germination of all seeds was almost completely inhibited in STS. Simultaneous application of ACC and STS enhanced germination of only 30°/20°C at 36°C in dark.

The effects of ACC, STS, and combinations of both on germination of seeds matured at 20°/10°C and 30°/20°C demonstrate the complexity of lettuce germination. Application of 10 mM ACC strongly increased germination of all seed lots at 36°C in light and of 30°/20°C seeds in dark. This served as evidence that fewer lettuce seeds could meet the increased ethylene requirements at supraoptimal temperature than at optimal and providing more ethylene precursor allowed their better germination. Nascimento et al. (2004a) reported that EVE seeds produced significantly more ethylene when imbibed in ACC than when imbibed in water. Application of ACC did not improve either the final percent germinated seeds or the speed of germination in either continuous light or continuous dark. This suggests that lettuce germination at optimal temperatures requires low levels of ethylene. However, application of ACC improved the uniformity of germination of all seed lots at 20°C, in both light and dark, suggesting that the presence

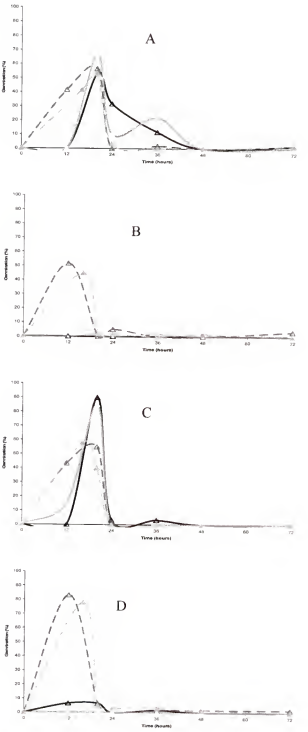


Figure 4-10 Uniformity of germination of DGB and EVE seeds matured at 20°/10°C or 30°/20°C during germination at 20°C or 36°C in continuous dark. Seeds matured at 30°/20°C were represented with broken lines while those matured at 20°/10°C were represented with solid lines. Open triangles represent EVE and filled triangles represent DGB. A) 20°C/water; B) 36°C/water; C) 20°C/ACC; D) 36°C/ACC; E) 20°C/STS; F) 36°C/STS; G) 20°C/ ACC + STS; H) 36°C/ ACC + STS.

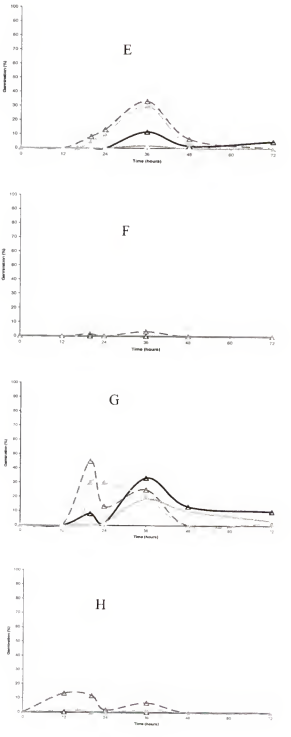


Figure 4-10.Continued.

of the ethylene precursor allowed more seeds from the population to produce ethylene and, consequently, to germinate earlier.

Both DGB and EVE seeds matured at 20°/10°C had only slightly higher germination at 36°C in the dark in ACC, although ACC increased their germination to above 70% at 36°C in light. This suggests that seed lots matured at 20°/10°C have a lower ability to convert ACC to ethylene in dark than in light which suggests that the activity of ACC oxidase (the enzyme converting ACC to ethylene) may be enhanced in light. There are no reports about the effect of light on expression of ACC oxidase genes or activity of ACC oxidase proteins in any plant species. There are several reports about stimulation of ACC synthesis in light. Sunohara et al. (2003) reported that ACC synthase activity was strongly enhanced in pesticide-treated maize seedlings in light but not in dark. Bessler et al. (1998) hypothesized that the light-stimulation of ethylene production in *Tillandisia usneoides* was through increasing the amount of available ACC. It is possible that light stimulates the expression of lettuce ACC oxidase gene(s) or the activity of ACC oxidase protein(s) indirectly. Gallardo et al. (1992) reported that *in vivo* ACC oxidase activity was inhibited by ABA in embryonic axes of chick-pea seeds. Light is known to inhibit ABA biosynthesis and stimulate ABA catabolism in seeds of many species, including lettuce (Roth-Bejerano et al.,1999). Therefore, light may enhance ACC conversion to ethylene in DGB and EVE seeds by counteracting the effect of some ACC oxidase inhibitor (potentially ABA). It is unknown why seeds matured at 30°/20°C can convert ACC to ethylene in dark at 36°C efficiently. Some of the possible explanations are that seeds matured at 30°/20°C have higher amounts of ACC oxidase or that their ACC oxidase does not require light stimulation. In order for the mechanism of light

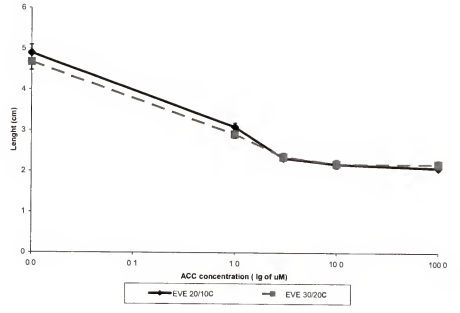
stimulation of ethylene biosynthesis to be established, the *in vivo* and *in vitro* activities of ACC synthase and oxidase in seeds matured at the two different temperatures during imbibition under different conditions need to be examined.

Application of 20 mM STS reduced germination of DGB and EVE seeds matured at either 20°/10°C and 30°/20°C in a similar way at 20°C in continuous light and dark. This indicates that there is a similar requirement for ethylene in both light and dark at the optimal temperature. ACC may partially negate the effect of STS by providing more ethylene to bind to the ethylene receptors prior to the displacement of the copper ions by silver ions.

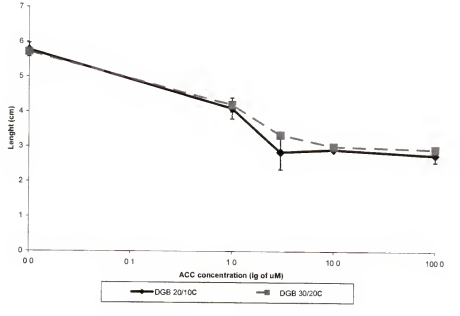
The mechanism of the promotive effect of ethylene on lettuce germination at supraoptimal temperature is unknown. Several hypotheses have been suggested. Abeles (1986) observed swelling of the hypocotyls of 'Grand Rapids' lettuce seedlings after exposure to ethylene and hypothesized that ethylene might participate in the development of osmotic potential in lettuce embryos prior to germination. Nascimento et al. (2000) correlated ethylene production with the activity of endo-beta-mannanase prior to lettuce germination. Promotive effect of ethylene on the activity of an enzyme involved in endosperm weakening in tobacco seeds, β-glucanase, has also been reported. It is possible that the promotive effect of ethylene on lettuce germination is indirect. *Arabidopsis* seeds having increased sensitivity to ethylene had lower sensitivity to ABA, while ethylene insensitive seeds had higher sensitivity to ethylene than wild-type *Arabidopsis* seeds (Beaudoin et al., 2000; Ghassemian et al., 2000). Thermoinhibition of lettuce seeds was reported to be accompanied by enhanced sensitivity to ABA (Gonai et al., 2004).

Lettuce germination depends not only on ethylene production but also on proper perception of ethylene. When ethylene perception is blocked by STS, germination of lettuce seeds is decreased (Chapter 3). In order to test whether the higher germination of seed lots matured at 30°/20°C is not due to enhanced sensitivity to ethylene, the ability of the four seed lots to respond to exogenously supplied ACC was tested using a triple response bioassay. During the experiments, the length of the primary roots and hypocotyls of DGB and EVE 20º/10ºC and 30º/20ºC seeds were measured. The decrease of length of the hypocotyls on increasing ACC concentrations was utilized to access the sensitivity to ethylene. The reduction in hypocotyl length has been used to determine differences in ethylene perception between ethylene insensitive and wild-type genotypes of lettuce and other plant species (Bleecker et al., 1988; Ecker and Guzman, 1990; Saltveit et al., 2003).

The reduction in hypocotyl length on increasing ACC concentrations of DGB and EVE seeds matured at different environmental conditions (30°/20°C and 20°/10°C) was similar (Figure 4-11). DGB hypocotyls, regardless of the temperature during seed maturation, were longer than EVE hypocotyls in absence of ACC. The most probable explanation for this was that there were some phenotypic differences between the two genotypes, unrelated to ethylene production or perception. Since DGB and EVE seeds produced similar amounts of ethylene at 20ºC in dark (the conditions of the triple response), it was unlikely that the differences in their hypocotyl lengths were due to differences in ethylene production.



A



B

Figure 4-11. Reduction of hypocotyl length (triple response bioassay) of EVE and DGB seedlings matured at 20º/10ºC or 30º/20ºC grown at 20ºC for 10 days in dark. A) EVE. B) DGB.

The triple response bioassay experiments indicated that the differences in the germinability of DGB and EVE seed lots matured at 20°/10°C or 30°/20°C were the result of differences in ethylene production and not ethylene perception.

Summary

Germination of both the thermosensitive genotype 'Dark Green Boston' (DGB) and the thermotolerant genotype 'Everglades' (EVE) at supraoptimal temperatures can be improved by maturation of the seeds at temperatures of 30°/20°C versus temperatures of 20°/10°C. Seed development and maturation at 30°/20°C improved germination at supraoptimal temperature in both light and dark and, also, germination in the ethylene action inhibitor, silver thiosulfate (STS), at both optimal and supraoptimal temperatures, in both continuous light and dark. DGB and EVE seeds matured at 30°/20°C produced more ethylene during germination than seeds matured at 20°/10°C, regardless of the imbibition conditions. Ethylene production correlated with germination in all seed lots, under all imbibition conditions. When DGB and EVE seeds matured at both 30°/20°C or 20°/10°C were imbibed in 10 mM 1-aminocyclopropane-1-carboxylic acid (ACC), their germination at supraoptimal temperature in both light or dark was significantly increased as compared to that in water. Application of ACC also negated to some extent the effect of STS on germination. Seeds matured at 30°/20°C and 20°/10°C did not have different sensitivity to ethylene. These results suggest that the ability of lettuce seeds to germinate at supraoptimal temperature is related to their ability to maintain and increase their ethylene production at supraoptimal temperature.

CHAPTER 5
MOLECULAR AND PHYSIOLOGICAL CHARACTERIZATION OF TRANSGENIC LETTUCE LINES WITH REDUCED GERMINATION

Introduction

Development of transgenic plants with altered biochemical and physiological processes has become one of the most powerful tools of modern molecular biology and plant physiology. The use of transgenics provides significant benefits when compared to the use of chemical inhibitors, mutants or recombinant lines. Expression of single genes can be altered in transgenics to overcome biotic or abiotic stresses.

A significant amount of information has been gathered regarding ethylene biosynthesis and perception, which has allowed development of transgenic lines that differentially produce or perceive ethylene. Ethylene biosynthesis starts with production of 1-aminocyclopropane-1-carboxylic acid (ACC) and 5-methylthioadenosine from S-adenosylmethionine via ACC synthase. The 5-methylthioadenosine is converted via the Yang cycle back to S-adenosylmethionine. The existence of the Yang cycle allows the levels of methionine to remain constant even during high ethylene production. ACC is further converted to ethylene via the enzyme ACC oxidase. Both ACC synthase and ACC oxidase are present as multigene families (isogenes) in all plant species studied so far and subject to complex transcriptional and posttranscriptional regulation ensuring proper ethylene production. Alterations in the expression of some of ACC synthase

or oxidase isogenes in transgenic plants has led to alterations in specific ethylene-mediated plant physiological processes. In order to reduce the amount of ethylene produced in tomato, Oeller et al. (1991) antisensed one of the tomato ACC synthase genes. The resulting transgenic tomato fruits had a 99.5% reduction in ethylene production and did not ripen. For the same purpose, Hamilton et al. (1990) antisensed one of the tomato ACC oxidase isogenes and obtained tomato plants with lower ethylene production.

Another approach to manipulate ethylene is through introduction of foreign, usually bacterial or bacteriophage enzymes, that can degradate ACC or its precursor, S-adenosylmethionine (SAM). Attempts to introduce three such enzymes, ACC deaminase, SAM decarboxylase and SAM hydrolase have been made. ACC deaminase is an enzyme present in soil microorganisms that can convert ACC to ammonia and α-ketobutyrate. By inserting it into tomato, Klee et al. (1991) were able to obtain fruits with delayed ripening. Expression of SAM-hydrolase, an enzyme from bacteriophage T3 that converts SAM to 5-methylthioadenosine and homoserine, reduced ethylene levels in transgenic tomato fruits (Good et al., 1994). Expression of SAM decarboxylase did not result in a reduction of ethylene production in potato plants due to the higher affinity of ACC synthase for SAM over that of SAM decarboxylase. The strongly expressed SAM decarboxylase could not channel more ACC towards polyamine synthesis, which might have resulted both in altered ethylene biosynthesis and polyamine biosynthesis in these transgenic plants. Araki et al. (2000) expressed bacterial ethylene-forming enzyme in tobacco and obtained transgenic plants with increased ethylene production and a dwarf phenotype that resembled the phenotype of plants exposed to exogenous ethylene.

Since there is less information about ethylene perception than about ethylene biosynthesis, less work has been done to obtain transgenic plants with reduced ethylene sensitivity. Several groups have expressed mutant *Arabidopsis thaliana etr 1-1* genes or chimeric *etr 1-1: Nr* genes in different plants species, obtaining tomato, petunia and carnations flowers with strongly reduced or completely inhibited ethylene perception (Wilkinson et al., 1997; Bovy et al., 1999). The researchers observed pleiotropic phenotypic effects of the ethylene insensitivity, including delay of flower senescence, inhibition or delay of fruit ripening and inhibition of lateral roots formation (Wilkinson et al., 1997; Bovy et al., 1999; Clark et al., 1999). Ethylene insensitive transgenic plants were also obtained by introduction of mutant genes presumed to be located downstream in the ethylene signal transduction pathway (*ein*). However, the level of ethylene insensitivity obtained was lower than that obtained through introduction of mutant ethylene receptors (Barry, 2004).

Lettuce (*Lactuca sativa* L.) is one of the plant species in which transgenic plants have been successfully created (Torres et al., 1993; Curtis et al., 1999b). Curtis et al. (1999b) expressed chimeric nitrate reductase gene in lettuce in order to reduce nitrate levels in the leaves. Jain and Nessler (2000) tried to engineer an alternative pathway for ascorbic acid biosynthesis in lettuce. Another area of gene engineering in lettuce has been that of herbicide resistance. McCabe et al. (1999b) obtained T3 lettuce plants with stable resistance to the herbicide glufosinate ammonium.

There has been no work on altering plant hormone biosynthesis or perception in lettuce. The only exception is the work of Curtis et al. (1999a) in which an *Agrobacterium tumefaciens* gene encoding an enzyme involved in the initial stages of

cytokinin biosynthesis was introduced in the lettuce cultivar 'Saladin'. Since several endogenous promoters having different types of deletions were used, the transgenic shoots exhibited different phenotypes, including gall production, dwarfism, vitrification and altered rooting. Unstable T-DNA expression in transgenic lettuce plants was reported (Falk, 1996; Gilbertson, 1996). The exact reasons for this instability were not known. They might have been related to the use of constitutive promoters (McCabe et al., 1999b) or to inactivation of the transgene following meiosis between T0 and T1 generations (McCabe et al., 1999a).

Our attempts to produce transgenic lettuce lines with reduced ethylene perception or altered ethylene production were aided by the development of a reliable system for transformation of iceberg lettuce cultivars in the years 1980-1990. Torres et al. (1993) successfully transformed 'South Bay' iceberg lettuce with vector containing the GUS gene and a gene for kanamycin resistance. The progeny of six independent T0 plants had segregation ratios of kanamycin resistant: kanamycin susceptible seedlings close to 3:1 and the inheritance of the kanamycin resistance were always linked to GUS activity. This suggested that stable inheritance of a transgene in lettuce is possible. In 1999, Torres et al. observed that leaves of transgenic greenhouse grown lettuce plants had increased resistance to glyphosate as compared to wild-type leaves. The same authors reported that the segregation of second generation transgenic seedlings was also 3:1 for GUS activity. The subsequent generations of glyphosate resistant 'South Bay' plants were tested for glyphosate resistance under field conditions and stable expression of the transgene was confirmed (Nagata et al., 2000).

The objective of the present work was to create transgenic lettuce lines with reduced ethylene perception or altered ethylene production in order to better understand the role of ethylene in lettuce germination. Studies with such lines will help determine the extent to which ethylene perception and production are important for germination of thermosensitive and thermotolerant lettuce genotypes at optimal and supraoptimal temperatures.

Materials and Methods

Plant Material

Seeds from the thermosensitive genotype 'Dark Green Boston' (DGB) and the thermotolerant genotype 'Everglades' (EVE) were used. The seeds were produced, stored and cleaned as described in Chapter 3.

For transformation, seeds were wrapped in cheesecloth squares, 10 X 10 cm, and disinfested in 20% commercial bleach solution containing 2 drops of Tween 20 per 100 ml. After disinfestation, the solution was decanted and the seeds were rinsed three times with sterile ultra-pure water. Seeds were then placed on a filter paper platform (Whatmann #3 filter paper) in a sterile 250 ml Erlenmeyer flask with the base immersed in 30 ml sterile nutrient solution containing 2.15 g/l MS salts (Murashige and Skoog, 1962). Seedlings were grown at a constant temperature of 25°C, with 14 hours of light and 10 hours of darkness in growth rooms. Cotyledons were excised from 24-hr-old EVE seedlings or 48-hr-old DGB seedlings and used as explants for transformation.

Agrobacterium Strain, Vectors and Bacterial Growth Conditions

Agrobacterium tumefaciens ABI strains harboring either pMON 26601 or pMON 11054 vectors were used. Both vectors contained the *CP4syn* gene from *Agrobacterium tumefacien*s encoding a mutated form of 5-enolpyruvyl shikimate 3-phosphate (EPSPS) gene under the control of its endogenous promoter with preserved chloroplast targeting signal sequences. Torres et al. (1993) reported that introduction of this gene in 'South Bay' lettuce conferred resistance to the herbicide glyphosate. In addition to the *CP4syn* gene, pMON 26601 contained a hybrid gene between the *Arabidopsis thaliana etr 1-1* and tomato *NR* genes, which has been reported to confer ethylene insensitivity (Wilkinson et al., 1997) and pMON 11054 contained the tomato ACC synthase 2 under control of a P-FMV promoter. The bacteria were grown on Luria Broth (LB) solid medium containing bacto tryptone (Fisher Scientific Co., Fairlawn, NJ, USA) at 10 g/l, bacto yeast extract (Fisher Scientific Co.) at 5 g/l and sodium chloride (Fisher Scientific Co.) at 10g /l to which 50 mg/l kanamycin (Sigma, St. Louis, MO, USA), 100 mg/l spectinomycin (Sigma) and 25 mg/l chloramphenicol (Sigma) and 15 g/l bacto agar (Fisher Scientific Co.) were added. The pH of the medium was adjusted to 7.5. Cultures for transformation were inoculated in 250 ml Erlenmeyer flasks containing 50 ml liquid LB medium with the same concentrations of kanamycin, spectinomycin and chloramphenicol as those used for the solid medium. The inoculated flasks were placed on an orbital shaker, at 150 rpm and grown at 25°C for 16-20 hours until A_{600}=0.6-1.2 was obtained.

Plant Transformation

Cotyledons were dipped in an undiluted bacterial suspension for 3 minutes, then blotted two times with sterile filter paper (Whatmann #3) and cocultivated for 48 hours on shoot initiation medium containing 4.3 g/l MS salts, 30 g/l sucrose, 0.1 g/l myoinositol, 0.001 g/l thiamine HCl, 0.05 mg/l pyridoxine HCl, 0.05 mg/l nicotinic acid and 0.002 g/l glycine supplemented with 0.05 mg/l naphtaleneacetic acid (NAA) and 0.05 mg/l benzyladenine (BA). All chemicals were obtained from Sigma. The pH of the medium was adjusted to 5.7. After cocultivation, the explants were immersed twice for 10 minutes in 3 % sucrose supplemented with 0.1 g/l cefotaxime (Phytotechnology, Overland Park, KS, USA). Afterwards the explants were transferred to selection shoot initiation medium containing in addition to the compounds mentioned above also cefotaxime at 0.1 g/l and glyphosate at 50 μM. Following shoot initiation, shoots were transferred to shoot elongation medium that had the same composition as the shoot initiation medium with the replacement of NAA by isopentenyladenine (2ip) (Sigma) at 0.1 mg/l and zeatin at 0.1 mg/l. Explants were transferred to new initiation and elongation media every 14 days to ensure constant selection pressure. The transformed cotyledons were kept on shoot initation medium for a total of 42 days. Once shoots were 1 cm in height, they were transferred to rooting medium containing 2.15 g/l MS salts, 10 g/l sucrose, 0.1 g/l myoinositol, 0.001 g/l thiamine HCl, 0.05 mg/l pyridoxine HCl, 0.05 mg/l nicotinic acid and 0.002 g/l glycine, solidified with 7 g/l phytagar (Fisher Scientific Co.) and pH of 5.7. Before the stems were placed on rooting medium, they were dipped in 0.25 mg/ml indolylbutyric acid (IBA) (Sigma). During subsequent transfers, IBA concentration was reduced to 0.125 mg/ml then eliminated.

Plant Growth Conditions

After the plantlets (designated as T0 generation) had developed at least two to three roots longer than 2 cm, they were transferred to soil. The explants were transferred from *in vitro* conditions to clam shell containers (18 X 8 cm), filled with 70% peat-moss: 30% vermiculate medium (Verlite Co., Tampa, FL, USA). The clam shell containers were placed inside a Lab-Line Instruments, Inc. (Melrose Park, IL, USA) incubator at ~ 25°C, 16-hr day/8-hr night photoperiod and light intensity of 100 μmol m^{-2} sec^{-1}. Plantlets were kept tightly closed for a period of at least one week, after which the containers were kept open for increasing periods of time. The soil was thoroughly moistened. Once the plantlets were able to grow in an open container, they were fertilized every other day with 7 g/3.3 l water of Peter's professional All Purpose Plant food. After the explants had adjusted to *in vivo* growth conditions (their leaves and root system had expanded), they were transferred to 3.78 l pots (Nursery Supplies, Inc., Kissimmee, FL, USA), filled with a mixture of 3:1 Transplant Mix A (Verlite Co.) and Horticultural Perlite (Aerosoil, Chemrock Inc., Jacksonville, FL, USA). Transfer from *in vitro* growth conditions to soil was a critical step in order to obtain transgenic plants. About 50% of the transferred plants died, regardless of the genotype or construct used for transformation. The reason might have been the inability of some plants to develop root system sufficient to provide all necessary nutrients or to withstand the low humidity in *in vivo* environment. Once established in pots, plants grew normally. The putative transgenic plants were grown at the Horticultural Sciences Department growth chamber facilities, in Convirons E-15 or walk-in chambers (Vollrath, River Falls, WI, USA) at 12

hr day/night, light intensity of 200 μmol m^{-2} sec^{-1} and temperatures of 25-30°C day and 18-20°C night.

Polymerase Chain Reaction (PCR) Analysis of Putative Transgenic Plants

DNA was extracted from young leaves using a modified Doyle and Doyle (1987) procedure, named DNA microprep for tomato/CTAB. When DNA purity was insufficient for the PCR reaction to proceed, DNA was subsequently extracted using the DNeasy Plant Mini Kit (Qiagen Inc, Valencia, CA, USA) according to the manufacturer's instructions. Genomic template DNA of 1, 4, or 6 μl DNA was used for each PCR reaction. The reaction mixture consisted of 25 mM $MgCl_2$, 10X PCR buffer, dATP, dCTP, dGTP, dTTP, 4 X 10 pmol/μl primers and Taq polymerase (Promega U.S., Madison, WI, USA). The primers used for amplification of the *CP4syn* gene were: 5' CCT TAG TGT CGG AGA GTT CG 3' and 5' CGG TGC AAG CAG CCG TCC AGC3'. The primers used for amplification of the endogenous lettuce gene were: 5' CGA CCT CCC TTT GCA AGA TA 3' and 5' TCA ACA GTT CGC TCA ATT CG 3'. The PCR reaction was carried out in a DNA Thermal Circler 480 (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95°C (1 min), 53°C (2 min), and 72°C (1 min) for 40 cycles. The PCR reaction products were analyzed using 1.0% (w/v) agarose (Fisher Scientific Co.) gels with incorporated ethidium bromide that were electrophoresed at 180 V (3-4 hours) and viewed under UV-illumination. During each PCR assay, both a negative control (DNA isolated from nontransformed lettuce plants) and a positive control (plasmid DNA or DNA from previously established glyphosate resistant plants) were included.

Southern Blot Hybridization

Approximately 20 µg total genomic DNA, extracted via a modified Dellaporta method was digested separately with both HindIII and EcoRV to confirm the presence of the T-DNA insert and to draw conclusions about the number of independent insertions. Lines obtained with pMON 26601 were abbreviated 'ETR' while the lines obtained with pMON 11054 were abbreviated 'ACC'. Leaf tissue was collected from three week old lettuce seedlings from homozygous T2 ACC lines and homozygous T3 ETR lines. Digestion with HindIII releases a 2 kb fragment corresponding to *CP4syn* plus its terminal sequence, and EcoRV cuts once inside the T-DNA and once outside of the transgene which allows independent insertions to be detected. Genomic DNA was digested for 5 hours at 37°C with 10U/µg HindIII and 15U/µg EcoRV, followed by electrophoresis on 0.8% agarose gel at 35 V overnight. The digested DNA was capillary transferred to Hybond N membrane (Amersham Biosciences Corp., Piscataway, NJ, USA) and bound by UV cross-linking. The membrane was probed with a 1.7 kb fragment corresponding to *CP4syn*. The probe was prepared directly from the construct. Plasmid DNA was isolated using a Plasmid Purification Maxi Kit (Qiagen, Inc.) and subjected to simultaneous double digestion with HindIII and BamHI (overnight, at 37°C) which resulted in release of 5 fragments, one of them corresponding to *CP4syn*. The probe was labeled using a random primed-labeling kit (Prime-a-gene Labeling System, Promega U.S.) and ^{32}P (PerkinElmer, Boston, MA, USA). The blot was pre-hybridized in a tube with 10 ml hybridization solution (7% SDS, 0.5M Na_2HPO_4) and 0.5 µg herring sperm DNA (Promega U.S.) at 65°C. Afterwards, the probe was added to 5 ml fresh hybridization buffer with 0.5 µg herring DNA heated at 65°C and added to the blot. The

blot was hybridized overnight, for approximately 18 hours. Then, the blot was washed with 70 ml 0.1X SSC with 0.1% SDS three times. The first wash was brief, followed by two 20 min washes. All washes were done at 65°C. After the third wash, the blot was removed from the tube, wrapped in saran wrap and tested with hand-held Geiger counter for signal. Then it was placed in X-ray film cassette and exposed to AR X-ray (Kodak X Omat) film for a week at -80°C.

Glyphosate Resistance Root Bioassay

In order to establish which transgenic lines were able to express the transgene, to identify the number of independent insertion events and to screen for homozygous plants, a modified glyphosate resistance root bioassay according to Torres et al. (2004) was used. Two (for testing T1 plants) or three (for testing T2 plants) replications, each of 20 seeds, were placed on medium containing 4.3 g/l MS salts and 0.2% phytagel (Sigma), pH 5.7. The medium was supplemented with 0, 100 or 200 μM glyphosate (Rodeo, Monsanto Co., St. Louis, MO, USA) for T1 plants and only 0 or 200 μM glyphosate for T2 plants. The glyphosate was added after the medium had been autoclaved and cooled. The medium was sterilized by autoclaving for 25 min at 100°C/1.1 kg/cm^2 pressure and then dispensed in phytotrays (Sigma). Seeds were sterilized with 20% commercial bleach solution for 30 minutes, followed by three times repeated rinsing with sterile water. Seedlings were grown at 25°C in 14/10 hr day/night for 13 days. On the 13th day, both radicle length and the number of secondary roots were measured and counted for each seedling.

Ethylene Sensitivity Assay

The sensitivity of DGB and EVE seeds with altered ethylene perception was examined as described in chapter 4. The range of ACC used was from 0 to 100 μM.

Ethylene Determination

The amount of ethylene produced by leaves and flowers was measured for T1 ACC plants. Ethylene was measured in two separate experiments (two replications each) for the leaves and in one experiment (three replications) for the flowers. In order to examine whether different temperatures affected the amount of ethylene produced, the plants were grown at two temperature regimes 30°/20°C or 25°/15°C. Replications consisted of both nonpollinated and pollinated flowers. Flower stems were cut about 1 cm below the petals and the cuttings were inserted into 1.5 ml microfuge tubes (Fisher Scientific Co.) containing 1.4 ml ultra-pure water. These, in turn, were placed inside 15-ml culture tubes, which were capped with rubber stoppers and incubated at room temperature until samples were taken. Ethylene production was measured at 1 and 4 hours after the start of incubation. For leaves, tubes were left open for one hour before capping to allow 'wound-induced' ethylene to escape. One ml sample was withdrawn using a gas-tight hypodermic 1 ml syringe (Fisher Scientific Co.). Ethylene was assayed using a gas chromatograph (Hewlett Packard Series II 5890) equipped with a flame ionization detector. The carrier gas was nitrogen, oven temperature was 130°C, injector temperature was 110°C, and detector temperature was 150°C.

Results and Discussion

Plant Transformation

Both DGB and EVE cotyledons were very amenable to plant transformation. It was observed that the youngest cotyledons produced the highest number of shoots. Consequently, 24-hr old and 48-hr old cotyledons were used for EVE and DGB, respectively. The reason older cotyledons were used for DGB was their slower germination and growth to attain the desired cotyledonary size. Cotyledon expansion was observed several days after placement on shoot initiation medium and sections (presumably transformed regions) turned green while other portions became yellow. Within 14 days, formation of callus was observed around the edges of the cotyledons. By 28 days, the cotyledons had expanded significantly and were covered with a lot of dense, regenerative type white or green callus. At the same time, small shoots could be observed that were moved to shoot elongation medium. After 42 days, almost all cotyledons were completely white or yellow and were discarded. The shoots were kept and grown on elongation media.

Since there was minimal callus development on shoot initiation medium, it was concluded that shoots were produced via predominantly direct organogenesis. The shoot initation medium used here was modified from Torres et al. 1993 in respect to the BA concentration. The latter was decreased from 0.2 to 0.05 mg/l to avoid excess callus production. For both DGB and EVE, the percent explants with shoots and the mean number of shoots per explant was significantly higher on 0.05 mg/l BA than on 0.2 mg/l BA. On explants from both genotypes, there was more callus on 0.2 mg/l BA than on

0.05 mg/l BA (Figure 5-1). In preliminary studies, a glyphosate concentration of 50 μM was found to be most suitable for both induction of the greatest number of shoots and a relatively high percentage transformed versus 'escape' (non-transformed) shoots.

Elongation of putative transgenic shoots was slow. Shoots had to be grown on elongation medium for at least 2.5 months prior to transfer to rooting medium. Possibly, the slow elongation was related to the presence of glyphosate in the medium. Selection pressure was found to reduce the speed of elongation in other plant species, such as melon (Nunez, personal communication). Shoots were formed in clusters and as they elongated, they were separated into singles. Most shoot clusters appeared to be chimeric; containing both green and white shoots, indicating that they did not originate from a single cell. Chimeric shoots with green and white regions were also observed but they were discarded.

Regeneration potential of lettuce cotyledons was dependent on the construct used. Cotyledons transformed with pMON 26601 produced more shoots than those transformed with pMON 11054. Arigita et al. (2003) reported that application of ACC inhibited organogenesis in kiwi explants while AVG increased the length and number of shoots obtained. Brara et al. (1999) also reported that the percent regeneration in cowpea explants increased significantly when their sensitivity to ethylene was reduced by addition of 50 μM $AgNO_3$ or 100 μM 2,5-norbornadiene. Cotyledons transformed with pMON 11054 also produced more callus than pMON 26601 cotyledons. In order to improve the morphogenetic potential of pMON 11054 cotyledons,

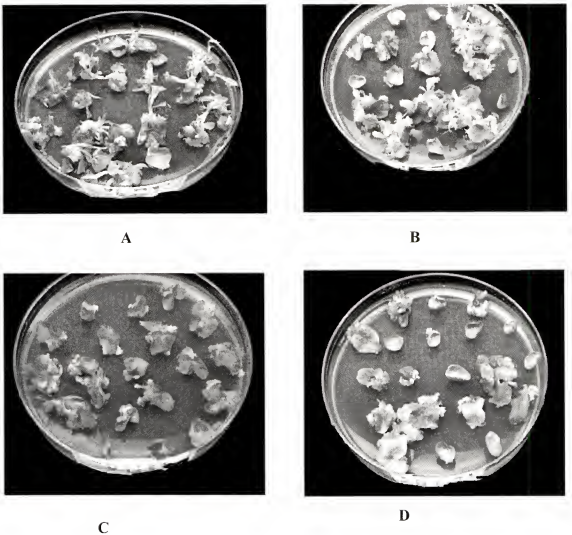


Figure 5-1 Nontransformed EVE and DGB 20-day old cotyledons on different BA concentrations. Both EVE and DGB explants produced more and longer shoots and less callus on media supplemented with 0.05 mg/l BA as compared to media supplemented with 0.2 mg/l BA. A) EVE, 0.05 mg/l BA. B) DGB, 0.05 mg/l BA. C) EVE, 0.2 mg/l BA. D) DGB, 0.2 mg/l BA.

they were placed on medium containing 50 μM silver thiosulfate or on standard medium but in the presence of 1.5 ml microfuge tubes containing potassium permanganate (K_2MnO_4). Although concentrations of STS up to 100 μM were noninhibitory for organogenesis in nontransformed cotyledons, there was no development of shoots from transformed cotyledons even on 50 μM STS. If pMON 11054 cotyledons were grown in the presence of K_2MnO_4, the number of explants with shoots and the mean number of shoots per explant were significantly greater than those for cotyledons grown on standard medium. Unfortunately, the cotyledons also produced significantly more callus due to use of phytotrays instead of Petri dishes, which led to accumulation of more moisture and stronger vitrification.

Even though DGB and EVE shoots were kept on elongation medium for at least 2.5 months, they elongated only slightly. When such explants were transferred to standard lettuce rooting medium (Torres et al., 1993), they did not produce roots and produced callus instead. Because of this, development of a more suitable medium was required. Even on a modified medium (with reduced concentrations of MS salts, sucrose and IBA), rooting took a long time (from two to four months) and required several transfers of the explants to fresh medium. During subsequent transfers the concentration of the IBA dip (0.25 mg/ml for the first medium) was gradually reduced. A gradual reduction prevented excessive callus formation. Only tall (higher than 3 cm) explants with well-developed stems were able to form roots (Figure 5-2). Shoots obtained from pMON 26601 transformations were more difficult to root than shoots obtained from pMON 11054 transformations because they formed roots more slowly.

DGB and EVE cotyledons had similar ability to give rise to shoots following transformation. For each, 7% of the cotyledons produced shoots. However, EVE was considered more amenable to transformation since the plantlets rooted better than DGB plantlets regardless of the construct used.

Polymerase Chain Reaction (PCR) Assay

Results from a PCR assay of 17 putative transgenic plants, including both EVE and DGB plants transformed with either pMON 26601 or pMON 11054, are represented in Figure 5-3. The upper, 1.3 kb band corresponds to a fragment from *CP4syn* while the lower, 0.9 kb band corresponds to an endogenous lettuce gene for methionine sulfoximide reductase, expected to be present in both wild-type and transgenic lettuce. A 4 µl DNA from each sample were used for each PCR reaction. Both the DNA extraction and the detection of PCR products were more difficult for DGB than for EVE. It is possible that DGB tissues contain more sugars or other compounds that decrease the purity of the DNA obtained and, consequently, influence the PCR assay.

Even though the PCR assay is useful to identify putative transgenic plants, it can render false negatives. When the PCR assay was conducted using samples of known and expressing glyphosate resistance plants, in some cases, the *CP4syn* gene was not detected, possibly due to insufficient DNA purity. However, since the numbers of plants identified as positive were enough for line development, the putative nontransgenic plants were discarded.

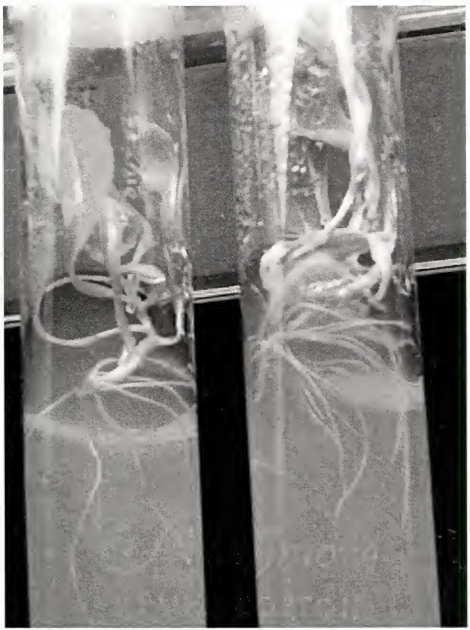


Figure 5-2 Successfully rooted putative DGB ETR plants

Glyphosate Resistance Root Bioassay

A root bioassay testing resistance of transgenic seedlings to exogenously applied glyphosate was used as a screen for identifying lines expressing the selectable marker (resistance to glyphosate). The use of such a test was important to determine the PCR positive lines that did not express T-DNA due to possible gene silencing.

Since seed populations from homozygous plants were needed for the studies, establishing the pattern of inheritance (single or double gene inheritance) was also important. This bioassay had previously been used by Torres et al. (2004) to examine the extent of glyphosate resistance among different lettuce transgenic lines carrying the *GUS* and the *CP4syn* genes. Modifications of this assay were also used for evaluation of glyphosate resistance in soybean seedlings and kanamycin resistance in lettuce seedlings (Torres et al., 2004). This bioassay is more accurate than the seedling assay and the whole plant assay developed by Nagata et al. (2000). The latter requires growing seedlings for a period of time in a greenhouse or growth chamber, which renders a seedling response to glyphosate greatly influenced by growth conditions, especially light.

In order to establish the pattern of inheritance of the transgene, three glyphosate concentrations were used, 50, 100, and 200 μM. Distinction between resistance and susceptibility to glyphosate was difficult on medium supplemented with 50 μM glyphosate because wild-type (WT) seedlings had similar lengths of primary roots and similar numbers of secondary roots as transgenic seedlings.

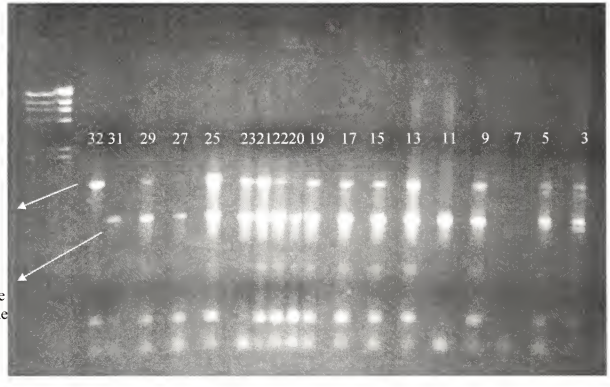


Figure 5-3 Identification of putative transgenic plants by polymerase chain reaction (PCR). Both the gene conferring resistance to glyphosate (*CP4 syn*) and the endogenous lettuce marker gene were detected in the putative transgenic plants while only the lettuce marker gene was detected in nontransformed and WT samples.

Lanes (from right to the left) 1,7,9,11,13,15,17,19,,20,21,22,27and 29: EVE ETR
Lane 23: DGB ETR
Lanes 3 and 5: DGB ACC.
Lane 31: WT EVE
Lane 32: tomato glyphosate resistant plant

Thus, 100 and 200 μM concentrations were needed to distinguish between resistant and susceptible seedlings. A concentration of 200 μM glyphosate was used for establishing the pattern of inheritance of the transgene.

For all three EVE ETR lines, the observed number of resistant and susceptible seedlings was not significantly different from the expected 3:1 ratio (Table 5-1). The primary roots of the resistant seedlings were longer than those of seedlings identified as susceptible. The primary root length of the resistant seedlings was similar for the three separate transgenic lines. For all three EVE ETR lines, seedlings identified as resistant had less secondary roots than seedlings identified as susceptible (Table 5-1).

On 200 μM glyphosate, resistant and susceptible seedlings were easily distinguished. The phenotype of glyphosate resistant seedlings resembled that of WT seedlings grown in absence of glyphosate (Figure 5-4). The primary roots were normal, long, and had numerous root hairs. The growth of the secondary roots started several days after germination. At the same time, the first true leaf appeared. About 10 days after germination, the second true leaf became visible. In susceptible seedlings, root growth was inhibited (the primary root was stunted, without root hairs and often no secondary roots). If there were secondary roots, they were without root hairs. No true leaves were observed in susceptible seedlings. Some had yellow cotyledons with necrotic zones. Since glyphosate affects plants via interfering with the aromatic amino acid biosynthesis by inhibiting the activity of a key enzyme in the shikimate pathway, the phenotype of the susceptible seedlings is a result of plant starvation.

Not all EVE ETR lines analyzed had clear distinctions between resistant and susceptible seedlings or segregation ratios of 3:1.

Table 5-1 Segregation ratios, average radicle length (cm) and average number of secondary roots for T0 EVE ETR lines grown for 13 days at 25°C on 200 μM glyphosate

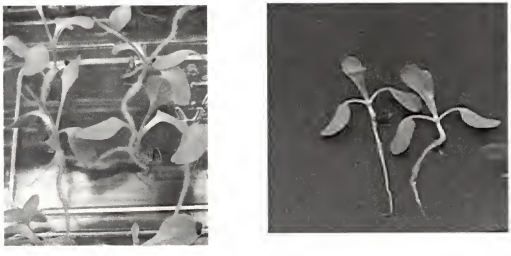
Transgenic Line	Resistant Seedlings		Susceptible Seedlings		X^2 for 3:1 Segregation
	Root length	# Secondary Roots	Root length	# Secondary Roots	
EVE ETR 2	2.64	1.6	0.49	0.8	2.84^{NS}
EVE ETR 6	2.52	0.7	0.45	0.0	0.67^{NS}
EVE ETR 18	2.80	1.3	0.60	1.0	0.01^{NS}

The data were analyzed using chi-square coefficient. * indicates significance at P=0.05 level and ** indicates significance at 0.01 level. **NS** stands for nonsignificantly different from the hypothesis 3:1.

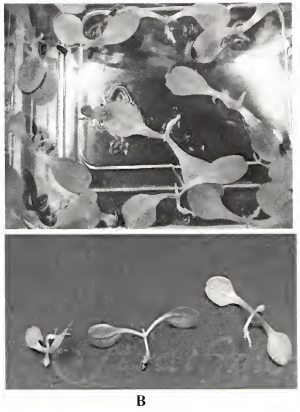
From the T1 transgenic lines analyzed, one line had no glyphosate tolerance and its phenotype was similar to WT. The PCR analysis of this line revealed no band corresponding to the *CP4syn* gene. Seedlings of four more lines, identified previously by PCR as positive, had very low tolerance to glyphosate. In presence of glyphosate, resistant seedlings from these lines had slightly longer primary roots than WT seedlings, more secondary roots (but lacking root hairs) and a short first true leaf. Possibly, the T-DNA was expressed very weakly in these lines due to insertion into a transcriptionally inactive genome region or due to partial posttranscriptional silencing. Such cases have been reported for other plant species like petunia (Meyer, 1995). One transgenic line (EVE ETR 24) had a segregation ratio close to 15:1 indicating a possible presence of two independent T-DNA insertions.

The segregation ratios, the primary root lengths, and the number of secondary roots were also measured for DGB ETR lines (Table 5-2). Segregation ratios for the three lines represented were similar to the EVE ETR lines, 3:1. The only exception was line DGB ETR 10 where the segregation ratio was significantly different from 3:1 on 200 μM glyphosate. However, since the segregation ratio for this line on 100 μM glyphosate was not different from 3:1, and the segregation ratio on 200 μM glyphosate was closer to 2:1 (not to 15:1 as is the segregation for two insertions), it was decided that this line still could be used for further experiments.

The mean length of the primary root of the resistant seedlings was similar for all three DGB ETR lines. In contrast to the EVE ETR lines, for DGB ETR lines, changes in the variable mean number of secondary roots appeared to be related to the classification of the seedlings as resistant or susceptible.



A



B

Figure 5-4 Phenotype of T2 EVE seedlings grown on 200 μM glyphosate at 25°C for 13 days. EVE ETR seedlings had long roots and visible first true leaves in contrast to WT EVE seedlings that had stunted roots and no first true leaf. A) Homozygous resistant EVE ETR seedlings. B) WT EVE seedlings.

The mean number of secondary roots for the susceptible seedlings of all DGB ETR lines was higher than that of the resistant seedlings. The general appearance of DGB ETR resistant and susceptible seedlings was similar to that of EVE ETR seedlings.

Since the number of DGB ETR lines tested was lower than that of EVE ETR lines (10 DGB ETR lines versus 23 EVE ETR lines) due to the difficult regeneration of plants from DGB cotyledons, less DGB ETR lines with different than 3:1 segregation and weak expression were found. One of the DGB ETR lines (DGB ETR 12) exhibited weak glyphosate tolerance similar to some of the EVE ETR lines. The seedlings of another DGB ETR line (DGB ETR 9) had no resistance to glyphosate and had negative PCR assay.

The mean length of the primary root, the mean number of secondary roots, and the phenology of resistant and susceptible EVE and DGB ACC seedlings were similar to the EVE and DGB ETR seedlings. Since there were fewer seeds from these lines, their segregation ratios and glyphosate tolerances were examined only on 200 μM glyphosate. No lines with segregation ratios different than 3:1 or with weak glyphosate tolerance were identified.

In conclusion, the glyphosate resistance root bioassay was necessary for identification of lines with strong transgene expression and probable presence of a single transgene, regardless of the genotype and construct used for transformation.

The mean length of the primary root and the number of secondary roots of T2 homozygous resistant seedlings from three (EVE ETR 2) and two (EVE ETR 6 and 18) individual plants were similar to those of T1 EVE ETR resistant seedlings (Table 5-3) indicating stability of expression.

Table 5-2 Segregation ratios, radicle length (cm) and number of secondary roots of T1 DGB ETR lines on 200 μM glyphosate

Transgenic Line	Resistant Seedlings		Susceptible Seedlings		X^2 for 3:1 Segregation
	Root Length	# Secondary Roots	Root Length	# Secondary Roots	
DGB ETR 6	3.35	0.7	0.51	2.1	2.97^{NS}
DGB ETR 10	3.22	0.9	0.67	2.1	5.10*
DGB ETR 14	3.68	0.5	0.55	1.2	0.18^{NS}

The data were analyzed using chi-square coefficient. * indicates significance at P=0.05 level and ** indicates significance at 0.01 level. **NS** stands for nonsignificantly different from the hypothesis 3:1.

The mean length of the primary root was significantly higher in all EVE ETR lines than in WT EVE seedlings. There were differences in the primary root lengths between the different plants and lines but they were not significantly different between lines. These small differences could be explained by variations in the environmental conditions which resulted in differences in seedling growth. In T2 EVE ETR generation, as in T1 generation, the number of secondary roots did not seem to be related to the transgenic character of the seedlings. The mean number of secondary roots was significantly higher in WT EVE seedlings than in seedlings of some EVE ETR lines (plants 2-5, 2-19, 2-20, 6-6 and 18-1) but not significantly different from other lines. The phenotype of the seedlings in general was similar to the T1 EVE ETR resistant seedlings and WT EVE seedlings grown in absence of glyphosate.

The lengths of the primary roots and the numbers of secondary roots were also measured for T2 homozygous DGB ETR seedlings (Table 5-4). Two control DGB lines were included in the experiments, WT DGB seedlings produced from plants not subjected to transformation and DGB ETR 9 seedlings, which came from one 'escape' plant. The primary roots of all DGB ETR seedlings were significantly longer than those of WT seedlings (Table 5-4). The primary roots of DGB ETR 6 seedlings were significantly longer than those of DGB ETR 14 seedlings which in turn were significantly longer than those of DGB ETR 10 seedlings. The variable mean number of secondary roots seemed less dependent on the resistant or susceptible character of the seedlings than the variable mean length of the primary roots (Table 5-4).

Table 5-3 Radicle length (cm) and number of secondary roots of T2 homozygous resistant EVE ETR seedlings on 200 μM glyphosate

Line	Radicle length (cm)	Secondary roots (#)
EVE ETR 2-5	2.77	0.63
EVE ETR 2-19	2.16	0.74
EVE ETR 2-20	2.35	0.98
EVE ETR 6-6	2.84	0.62
EVE ETR 6-7	2.56	1.54
EVE ETR 18-1	2.99	1.13
EVE ETR 18-5	2.74	1.66
WT EVE	0.51	1.88
LSD Value	0.37	0.51

Data were analyzed using ANOVA. Means were separated using Least Significant Difference (LSD).

Seedlings from the DGB ETR 14 line had the greatest mean number of secondary roots followed by DGB ETR 6 seedlings, WT DGB and DGB ETR 10 seedlings. Seedlings from the 'escape' line, DGB ETR 9, had a significantly greater number of secondary roots than any of the other lines allowing the seedlings to look 'healthier' than WT DGB seedlings , with greener cotyledons and visible first true leaf (phenotype characteristic of weak glyphosate resistance). This led to the hypothesis that this line expressed the *CP4syn* gene weakly. The phenotype of T2 DGB ETR seedlings from all other lines was similar to that of T1 resistant DGB ETR seedlings.

An interesting observation was made when the percent germinated seeds was counted 48 hours after the start of the experiment for both DGB ETR and WT DGB seeds. All DGB ETR lines germinated slower than WT DGB and reached 100% germination approximately 48 hours later than WT DGB seeds. Germination of ethylene insensitive DGB ETR seeds was significantly lower than germination of the two control seed populations (WT DGB and DGB ETR 9) at both 48 and 72 hours after the start of the experiment (Table 5-5).

Since the delayed germination of DGB ETR seeds was observed in both the presence and absence of glyphosate, it was not probable that this delay was a result of the glyphosate presence. Since such a delay in germination was observed neither in EVE ETR seeds nor in ACC seeds, it was probable that it was not caused by a decrease in seed vigor as a result of presence of the transgene. The delayed germination of DGB ETR seeds might be a result of their decreased sensitivity to ethylene and, as a consequence, lower ability to respond to its promotive effect even at optimal temperature (25°C) in light.

Table 5-4 Radicle length (cm) and number of secondary roots of T2 homozygous resistant DGB ETR seedlings on 200 μM glyphosate

Line	Radicle Length (cm)	Secondary Roots (#)
DGB ETR 6-2	3.93	0.98
DGB ETR 6-6	3.45	0.95
DGB ETR 10-8	2.77	0.65
DGB ETR 14-2	3.17	1.35
DGB ETR 14-3	3.30	1.10
DGB ETR 14-5	3.15	1.33
WT DGB	0.60	0.75
DGB ETR 9	0.75	2.09
LSD Value	0.53	0.36

Data were analyzed using ANOVA. Means were separated using Least Significant Difference (LSD).

Beaudoin et al. (2000) and Ghassemian et al. (2000) reported that *Arabidopsis thaliana* seeds with lower sensitivity to ethylene required a longer time to exit primary dormancy and germinate. Delayed germination was not observed in T1 glyphosate resistant DGB ETR seedlings, suggesting that strong ethylene insensitivity conferred by a homozygous state of the mutant gene might be required for this type of germination behavior to be observed.

The mean length of the primary roots and the mean number of secondary roots were also measured for both EVE and DGB ACC T2 homozygous resistant seedlings (Table 5-6). All ACC lines had significantly longer roots than the respective WT EVE or DGB seedlings and the control (azygous, meaning lacking the transgene and consequently glyphosate susceptible) EVE ACC 3-12 seedlings. The seedlings from the three individual DGB ACC 2 plants had similar mean primary root lengths. These lengths were significantly greater than those of EVE ACC seedlings. Seedlings of the three EVE ACC 3 plants had significantly shorter primary roots than the other two EVE ACC lines despite the similar rate of germination. All DGB and EVE seeds germinated at 90-100% during the first 24 hours of imbibition. It is possible that EVE ACC 3 seedlings had lower ability to grow than the seedlings from the other EVE ACC lines under the conditions of the glyphosate resistance root bioassay. The variable mean number of secondary roots was not significantly different between WT seedlings and ACC seedlings (Table 5-6).

In conclusion, the glyphosate resistance bioassay proved to be very useful for identification of homozygous resistant DGB and EVE plants with either reduced ethylene perception or altered ethylene production.

Table 5-5 Germination (in %) of T2 homozygous resistant DGB ETR seedlings and WT DGB seedlings at 25°C on 200 μM glyphosate

Line	Time (hours)		Final Percent Germination
	48 hours	72 hours	13 days
DGB ETR 6-1	21.7	76.7	93.3
DGB ETR 6-2	20.0	73.3	100.0
DGB ETR 6-6	20.0	65.0	100.0
DGB ETR 14-5	18.3	76.7	96.7
WT DGB	91.7	100.0	100.0
DGB ETR 9	63.3	93.3	100.0
LSD value	18.3	19.7	NS

Data were analyzed using ANOVA. The percents germinated seeds were separated using Least Significant Difference (LSD).

Glyphosate resistant seedlings, regardless of genotype or construct used for transformation, had significantly longer primary roots than glyphosate susceptible seedlings. In contrast, the number of secondary roots did not differ statistically between glyphosate resistant and susceptible lettuce seedlings. The latter observation is not consistent with the results of Torres et al. (2004) who reported that the number of secondary roots correlated well with resistance to either glyphosate or kanamycin. The discrepancy might be due to the use of a different genotype, 'South Bay' by Torres et al. (2004) that had much more secondary roots than either EVE or DGB in absence of glyphosate.

Phenotype of the Transgenic Plants

The phenotype of the first generation (T0) ETR plants obtained from tissue culture was different from that of lettuce plants growing from seed. First generation ETR plants were taller, had fewer leaves and did not form a head structure. They also flowered and produced seeds shortly after transfer to soil, which was attributed to the age of the tissue. However, these changes were not heritable since the second generation plants were 'true to type' and identical to WT plants. The morphology of the T1 ETR leaves, stems and flowers appeared normal. Flowering began approximately 3 months after sowing and seeds developed two weeks after pollination. There was no requirement for hand pollination. Each plant produced up to 5 g of seeds. ETR plants from both genotypes senesced slower than WT plants (ETR plants were completely senesced one month after WT plants).

Table 5-6 Radicle length (cm) and number of secondary roots of T2 homozygous resistant EVE and DGB ACC seedlings on 200 μM glyphosate

Line	Radicle Length (cm)	Secondary Roots (#)
EVE ACC 1-8	2.22	0.70
EVE ACC 3-6	1.71	0.00
EVE ACC 3-7	1.90	0.12
EVE ACC 3-13	2.12	0.09
EVE ACC 4-13	2.44	0.50
DGB ACC 2-4	2.64	2.37
DGB ACC 2-5	2.44	1.85
DGB ACC 2-7	2.55	1.91
WT EVE	0.51	1.88
WT DGB	0.58	1.25
EVE ACC 3-12	0.51	0.92
LSD Value	0.21	0.42

Data were analyzed using ANOVA. Means were separated using Least Significant Difference (LSD).

Decreased perception of ethylene has been associated with delayed leaf and flower senescence in ornamental crops like petunia (Wilkinson et al., 1997). Possibly, senescence was delayed in the ETR plants because of a reduction in ethylene sensitivity.

In contrast to ETR plants, the putative ACC overexpressing plants (T0 ACC) had phenotypes very different from those of WT plants. They were smaller than WT or ETR plants and had fewer leaves (Figure 5-5). Their leaf appearance was unique and did not resemble any known lettuce phenotype (Ryder, personal communication). Their leaves appeared 'flat' or curved inward, with an unusual oval instead of triangular shape (Figure 5-5). T0 EVE ACC plants had difficulty forming seeds. Three to four weeks following pollination, seed pods were formed but were either empty or contained two to three seeds. Hand pollination did not improve seed formation. Since there was no problem with seed formation in either WT or ETR plants, it was hypothesized that ACC plants might have a problem forming seeds due to the expected high ethylene production. Because of this, an ethylene action inhibitor, 1-methylcyclopropane (1-MCP) (Floralife, Inc. Walterboro, SC, USA) in concentration of 1 mg/ml was applied to the ACC plants for a period of 24 hours. Reversion of the unusual phenotype was observed as early as 8 hours after the beginning of the 1-MCP application, which confirmed that the unique phenotype might have been related to an increase in ethylene production, but no improvement of the seed development was observed. Seed development was improved near the end of the reproductive life of the plants, about four weeks after the start of flowering. Since it was observed that ethylene evaluation from leaves of ACC plants decreased with plant aging, it was suggested that the reduced ethylene production might have allowed the seeds to develop.

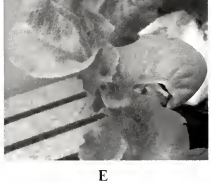
A

B

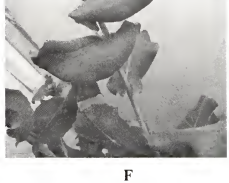
Figure 5-5 Comparison of general appearance and of leaf morphology of WT, ETR and ACC plants. A) WT EVE. B) EVE ACC. C) Close up of WT EVE. D) Close up of DGB ACC. E) Close up of EVE ACC. F) Close up of DGB ACC. Note that the EVE ACC plant is shorter, with fewer leaves. Note the difference in leaf appearance.

C

D



E



F

Figure 5-5 Continued.

The seeds themselves, especially from the lines with strongest phenotype EVE ACC 1 and 4, were smaller than WT and EVE ETR seeds, with irregular shape and appeared 'flat' which was suggestive of abnormal embryo development. Approximately 10% of the seeds had protruding radicles. Such phenotype has been observed in seeds of many plant species, deficient in or insensitive to ABA. Interactions between ABA and ethylene have been reported for both *Arabidopsis thaliana* and petunia seeds (Beaudoin et al., 2000; Ghassemian et al., 2000; Barry, 2004). Despite their abnormal appearance, EVE ACC seeds germinated 100% during glyphosate resistance root bioassays. However, their seedlings were smaller than WT and EVE ETR seedlings and often the radicles looked damaged.

DGB ACC plants developed seeds normally. Although their seed development was unaffected by putative production of more ethylene than WT plants, their senescence was affected T0 DGB ACC plants senesced more rapidly (several days compared to month) than WT or DGB ETR plants. In some lines, senescence was triggered earlier than expected naturally by small environmental stresses (plant wilting). Senescence of EVE ACC plants was more rapid than that of WT EVE plants but the difference was not as dramatic as for the DGB ACC plants.

The T1 homozygous resistant or heterozygous plants from three EVE ACC lines (numbers 1, 2, and 4) and two DGB ACC lines (1 and 2) had phenotypes identical to that of WT plants, along with normal seed development and senescence patterns. An exception was transgenic line EVE ACC 3 where plants had an abnormal phenotype and senesced prematurely. In plants of this line, seed development was delayed as in T0 plants. Based on the phenotype of T1 ACC plants, it was hypothesized that the ethylene

production in T1 ACC plants was suppressed not enhanced. In order to test this hypothesis, evaluation of ethylene production in T1 ACC plants was performed.

Triple Response Bioassay

The triple response bioassay is a method for screening for mutant or transgenic plants with altered ethylene perception. Bleecker et al. (1988) used a triple response bioassay to test a dominant mutation conferring ethylene insensitivity in *Arabidopsis thaliana*. Later, Guzman and Ecker (1990) used different variants of this screen with either ethylene or ACC to identify several new mutations in *Arabidopsis thaliana* affecting either ethylene production or ethylene perception. The triple response bioassay has been successfully used for identification of genes involved in ethylene signaling in various species such as tomato (Tieman et al., 2001) and geranium (Clark et al., 2001). There have been attempts with the cultivar 'Diana' to obtain mutant lettuce lines with reduced ethylene perception in an attempt to improve postharvest quality of mature lettuce heads (Saltveit et al., 2003). The authors conducted the triple response bioassay by exposing lettuce seedlings for 7 days to 10 µl/l ethylene and could detect ethylene insensitive mutants.

Partial insensitivity of DGB ETR lines to ethylene was observed as long hypocotyls on increasing ACC concentrations (Table 5- 7). For all transgenics, the average length of the hypocotyls did not decrease significantly on any ACC concentration as compared to the hypocotyl length in absence of ACC (Table 5-7). In contrast, wt DGB hypocotyls were significantly shorter on all ACC concentrations as compared to 0 µM ACC. This demonstrated that WT DGB seedlings had higher sensitivity to exogenous

ACC than DGB ETR seedlings which confirmed the partial ethylene insensitivity of the DGB ETR lines. DGB ETR 14 seedlings appeared to be more ethylene insensitive than DGB ETR 6 seedlings.

Even though the hypocotyls of DGB ETR seedlings were longer than those of WT DGB seedlings on all ACC concentrations, the differences were not statistically significant (Table 5-7). WT DGB hypocotyls were longer than DGB ETR hypocotyls in absence of ACC. The reason for this may be a slight inhibition of the ability of the transgenic seedlings to grow due to the presence of the T-DNA.

The hypocotyl length of all transgenic lines with the exception of EVE ETR 18-1 and WT EVE decreased significantly on 1 μM ACC as compared to no ACC (Table 5-8). EVE ETR 18-1 hypocotyl length decreased significantly on 10 μM ACC. EVE ETR seedlings from all transgenic lines had longer hypocotyls than WT EVE seedlings on 10 μM ACC (Table 5-8). On 1 μM ACC, EVE ETR 18-1 and EVE ETR 18-5 seedlings had significantly longer hypocotyls than WT EVE indicating that EVE ETR 18 line had the lowest ethylene perception of all lines. The data confirmed that the sensitivity of EVE ETR seedlings to exogenous ACC was lower than that of WT EVE.

In addition to the longer hypocotyls, ETR seedlings also had a smaller curvature of the apical hook, combined with thinner hypocotyls and longer roots (Figure 5-6). The wide range of differences between ETR and WT phenotypes confirmed that their longer hypocotyls were the result of actual reduction in sensitivity to ethylene.

Table 5-7 Triple response of WT DGB and T2 DGB ETR seedlings

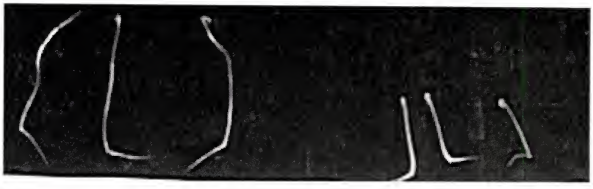
Hypocotyl Length (cm)					
ACC conc. (μM)	DGB ETR 6-1	DGB ETR 6-2	DGB ETR 14-2	DGB ETR 14-3	WT DGB
0	5.8	5.1	5.2	5.4	6.4
1	5.1	4.6	4.2	5.0	4.2
10	4.5	3.7	4.3	4.7	2.8
100	3.9	3.8	4.3	4.4	2.5
		LSD value	2.2		

Data were analyzed using ANOVA.

Table 5-8 Triple response of WT EVE and T2 EVE ETR lines

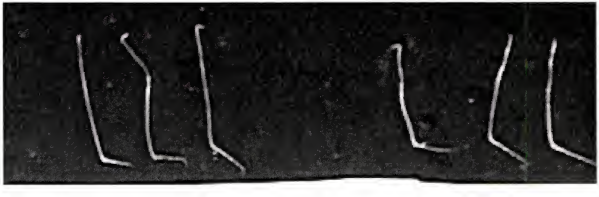
	Hypocotyl Length (cm)					
ACC conc. (μM)	EVE ETR 2-5	EVE ETR 6-6	EVE ETR 6-7	EVE ETR 18-1	EVE ETR 18-5	WT EVE
0	5.1	5.1	5.0	5.1	5.6	4.9
1	4.0	3.8	3.8	4.3	4.2	3.0
10	3.8	3.7	3.4	4.0	3.9	2.3
LSD value		1.0				

Data were analyzed using ANOVA.



EVE ETR 2

WT EVE



EVE ETR 18

EVE ETR 8

Figure 5-6 Difference of appearance of WT EVE and T2 EVE ETR seedlings grown at 20°C in dark for 10 days (triple response bioassay). Note the longer and appearing thinner hypocotyls and radicles of EVE ETR seedlings.

Southern Blot Analysis

Southern blots of the transgenic lines used for seed experiments were performed in order to provide more information about the number of independent T-DNA insertions. Samples digested with both HindIII (lanes 1-10) and EcoRV (lanes 11-20) are represented in Fig. 5-7. The amount of DNA loaded was not equal; however, it was sufficient to ensure complete digestion with both restriction enzymes and visible DNA low molecular weight fragments. The only partially digested line was #5 containing EVE ACC 3 DNA digested with HindIII. It was possible that the purity of the DNA used was lower than required for complete digestion since amplification of this DNA with different sets of primers during previous PCR experiments was not successful.

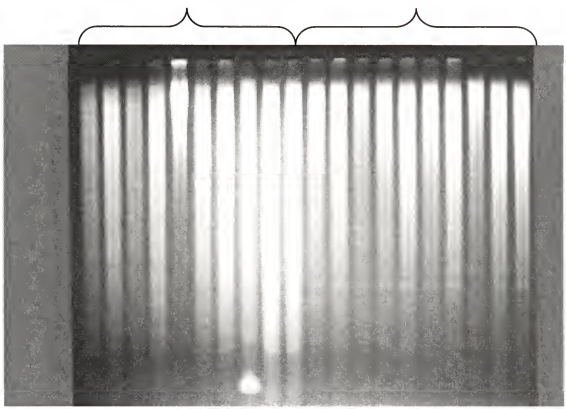
Autoradiography of the same DNAs digested with HindIII (the left part of the gel in Fig. 5-7) is represented in Figure 5-8 A. All transgenic samples revealed the presence of a ~ 2 kb fragment corresponding to the *Cp4syn* gene plus its terminator sequence. All the samples released the same molecular weight fragment. No signal was detected in WT EVE and DGB. The size of the hybridizing fragment was larger in melon DNA. The reason for this could be differences in the positions of the HindIII restriction sites. The signal was strongest in the DGB ETR 9 DNA, which was supposed to be an 'escape' line. The classification of this line as 'escape' was based on its negative PCR and lack of glyphosate resistance. However, the seedlings from this line had weak glyphosate tolerance suggesting that possibly multiple T-DNA copies were inserted in one location in the genome resulting in suppression of transgene expression. It has been reported that the chance of observing gene silencing increases with an increase in the number of T-DNA copies present (Meyer and Sadler, 1996). The same authors hypothesized that the

presence of several T-DNA copies in one location in the genome could lead to formation of condensed chromosomal complexes, similar to the repeat-induced heterochromatization process in *Drosophila melanogaster* that could not be accessed by transcription factors.

Hybridization of DNAs digested with EcoRV is represented in Figure 5-8 B. The size of the fragments was different for each transgenic line due to the insertion of the T-DNA in different locations. No signal was detected in DNA from WT plants. In DGB ETR 9 DNA, there was one strong signal detected, strengthening the suggestion that there might be multiple T-DNA copies in a single location in the genome of this line.

Ethylene Production of T1 ACC Plants

In order to establish whether the ACC transgenic plants produced more ethylene than WT plants, ethylene production *in vivo* by leaves and flowers of T1 ACC plants was measured. There were no significant differences between the amount of ethylene produced by leaves of ACC plants and WT plants (Tables 5-9, 5-10). There were no differences between the amounts of ethylene produced at the two plant growth conditions (30°/20°C versus 25°/15°C). The ethylene production in all plants regardless of growth temperatures was higher at one hour after the start of the experiment than at four hours. The reason for this may be the remaining production of 'wound-induced' ethylene at one hour despite the preceding ventilation of the samples. The inability to detect any differences in ethylene production between the lines might have been related to the effect of plant health and extent of wounding during sample collection on ethylene production.



Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 5-7 Digestion of genomic DNA from ETR and ACC seedlings with either HindIII or EcoRV

Lanes (from left to right): 1 and 11 EVE ETR 2 Lanes: 2 and 12 EVE ETR 6 Lanes: 3 and 13 EVE ETR 18
Lanes: 4 and 14 EVE ACC 1 Lanes: 5 and 15 EVE ACC 3 Lanes: 6 and 16 DGB ACC 2
Lanes: 7 and 17 DGB ETR 9 Lanes 9 and 19 Wt EVE Lanes: 10 and 20 Wt DGB
Lanes: 8 and 18 PCR+ melon plant

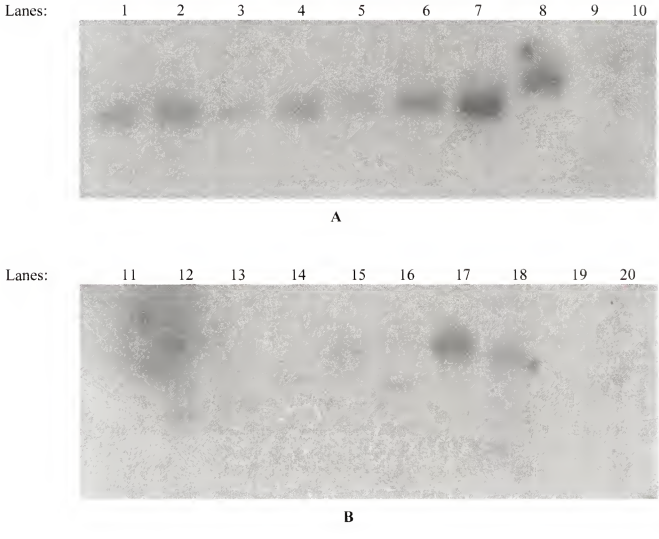


Figure 5-8 Detection of CP4syn gene using southern blotting hybridization of DNAs digested with A) HindIII. B) EcoRV.

Lanes (from left to right): 1 and 11 EVE ETR 2 Lanes: 2 and 12 EVE ETR 6 Lanes: 3 and 13 EVE ETR 18
Lanes: 4 and 14 EVE ACC 1 Lanes: 5 and 15 EVE ACC 3 Lanes: 6 and 16 DGB ACC 2
Lanes: 7 and 17 DGB ETR 9 Lanes 9 and 19 Wt EVE Lanes: 10 and 120 Wt DGB
Lanes: 8 and 18 PCR+ melon plants

Table 5-9 Ethylene production by leaves of T1 EVE ACC plants and WT EVE plants

Transgenic Line	Seed Developmental Temperature			
	30°/20°C		25°/15°C	
	Time (hours)		Time (hours)	
	One	Four	One	Four
EVE ACC 3	9.53 ±1.4	3.79 ±0.1	11.54 ±2.1	3.45 ±0.7
EVE ACC 4	16.84 ±11.2	3.45 ±0.5	8.96 ±3.1	3.16 ± 0.6
WT EVE	11.37 ±4.9	3.30 ±0.0	15.18 ±15.0	3.17 ±0.4

Ethylene production values were separated using Standard errors (SE).

Table 5-10 Ethylene production by leaves of T1 DGB ACC plants and WT DGB plants

Transgenic Line	Seed Developmental Temperature			
	30°/20°C		25°/15°C	
	Time (hours)		Time (hours)	
	One	Four	One	Four
DGB ACC 1	8.89 ±1.8	2.79 ±0.2	8.23 ±0.5	3.08 ±0.2
WT DGB	2.28 ±0.6	1.76 ±0.4	5.29 ±0.3	8.91 ± 8.8

Ethylene production values were separated using Standard errors (SE).

Since it was not possible to detect differences between WT and ACC plants by measuring *in vivo* leaf ethylene production, an alternative approach was sought. A decision was made to measure *in vivo* ethylene production in nonpollinated and pollinated flowers of WT and ACC plants. It is known that pollination induces a strong increase in the amount of ethylene produced in flowers of different vegetative (tomato) and ornamental (petunia, dendrobium) species (Ketsa and Rungkong, 2000; Llop-Tous et al., 2000). It was speculated that pollination might result in different amounts of ethylene produced by flowers of WT and ACC plants.

Nonpollinated flowers did not produce ethylene in either WT or ACC plants. The only exception were EVE ACC 3 flowers from plants grown at 30°/20°C, which produced 3.37 nl/g/h at one hour after the start of the experiment and 2.02 nl/g/h at four hours. Pollinated flowers from EVE ACC 3 plants produced more ethylene than WT EVE flowers at both 25°/15°C and 30°/20°C at both 1 hour and 4 hours after pollination (Table 5-11). In contrast, pollinated flowers from EVE ACC 4 plants produced significantly less ethylene than WT EVE flowers also under all experimental conditions. Flowers from DGB ACC 1 plants produced lower or similar amounts of ethylene than WT DGB flowers (Table 5-12). WT DGB flowers produced more ethylene at 30°/20°C than at 25°/15°C. This suggests that at least flowers from one genotype (DGB) were capable of producing more ethylene at higher environmental temperatures.

The ethylene production *in vivo* by flowers of T1 ACC plants was expected based on their phenotype. EVE ACC 4 and DGB ACC 1 plants that had a similar phenotype to WT plants produced similar or lower amounts of ethylene compared to WT plants.

Table 5-11 Ethylene production by pollinated flowers of T1 EVE ACC plants and WT EVE plants

Transgenic Line	Seed Developmental Temperature			
	30°/20°C		25°/15°C	
	Time (hours)		Time (hours)	
	One	Four	One	Four
EVE ACC 3	53.99 ±11.0	27.47 ± 8.6	10.34±2.9*	9.04 ±1.9*
EVE ACC 4	23.87±1.2	13.35 ±1.9	1.85 ±3.7 *	4.06 ± 0.8 *
WT EVE	41.03 ±5.1	18.46 ±2.1	4.07 ±5.8 *	6.20 ±2.5 *

The differences among the different ethylene values were demonstrated using standard error (SE). * indicates flowers in which the ethylene production was measured 24 hours after pollination due to lack of unopened flowers.

Table 5-12 Ethylene production from pollinated flowers of T1 DGB ACC plants and WT DGB plants

Transgenic Line	Seed Developmental Temperature			
	30°/20°C		25°/15°C	
	Time (hours)		Time (hours)	
	One	Four	One	Four
DGB ACC 1	48.65 ±20.9	21.31 ±17.6	37.08 ±13.5	28.18 ±12.6
WT DGB	95.63 ±3.2	43.80 ±3.2	25.52 ±6.7	29.01 ± 3.0

The differences among the different ethylene values were demonstrated using standard error (SE).

EVE ACC 3 flowers produced significantly more ethylene that WT EVE flowers and EVE ACC 3 plants had an abnormal phenotype. Based on this observation, it can be suggested that the abnormal phenotype is related to increased ethylene production. Since all T0 ACC plants had an abnormal phenotype, it could be speculated that they also produced high amounts of ethylene and that their ethylene production might have been suppressed in the T1 generation. In order to establish with certainty that this had happened, *in vitro* or *in vivo* ACC synthase activity or ACC synthase expression would have to be measured in T1 DGB and EVE ACC plants and WT plants.

The exact reasons for this suspected suppression are unknown. Since the Southern blot analysis reveals the presence of the T-DNA in T1 ACC plants, it is possible that the ACC synthase gene present in the T-DNA has been suppressed either transcriptionally or posttranscriptionally. It may be speculated that the strong effect of the suspected increased ethylene production in T0 ACC plants on their development might have triggered silencing of the trasngene. According to one of the existing hypothesizes about the mechanisms of posttranscriptional silencing, proposed by Meins and Kuntz in 1995, the presence of a transgene influences the action of endogenous genes with high homology to it. Strong trasngene expression may lead to suppression of the transgene and the endogenous, homologous to it, genes.

The amounts of ethylene produced by pollinated flowers of T1 ACC plants indicated the way the ethylene production was affected in these transgenic plants and how their germination might possibly be affected.

Summary

The work presented in this chapter describes the process of development of transgenic lettuce lines with either reduced ethylene perception or altered ethylene production. Transgenic plants from both the thermosensitive genotype 'Dark Green Boston' (DGB) and the thermotolerant 'Everglades' (EVE) were obtained using *Agrobacterium*-mediated transformation with lettuce cotyledons used as explants. The presence of the desired genes was confirmed using the polymerase chain reaction (PCR) assay and Southern blotting hybridization. Homozygous seed populations required for establishing the effect of reduced ethylene perception or altered production on germination were selected using a glyphosate resistance bioassay. The triple response bioassay identified several DGB and EVE lines with reduced ethylene perception. If perception of ethylene is required for germination, their germination will be affected at optimal or supraoptimal temperatures, or both. Using *in vivo* determination of ethylene in flowers of T1 ACC plants, it was established that EVE ACC 3 plants were capable of producing more ethylene than WT EVE plants while the production of all other T1 ACC lines was reduced as compared to WT. If the amount of ethylene produced influences lettuce germinability, it can be expected that ACC seeds will have increased germination if ethylene production is increased or reduced if ethylene production is reduced.

CHAPTER 6
REDUCED LETTUCE GERMINATION IN RESPONSE TO REDUCED ETHYLENE PERCEPTION OR PRODUCTION

Introduction

A role for ethylene in seed germination of different plant species continues to be controversial. In part, a reason for this controversy has been the use of ethylene inhibitors that potentially influence unrelated to ethylene processes in seeds. Recently, availability of mutants and use of transgenic plants with altered ethylene perception or biosynthesis has increased interest in studying ethylene effects on germination.

The first evidence for a role of ethylene in germination came from work with mutant *Arabidopsis thaliana* plants (Bleecker et al., 1988). Freshly harvested *etr 1-1 Arabidopsis* seeds did not germinate even following chilling at 4°C for 3 days. The same treatment resulted in higher than 95% germination of wild-type (WT) *Arabidopsis thaliana* seeds. However, *etr 1-1* seeds had greater than 90% germination if treated with 5 μM GA_3 during chilling. Beaudoin et al. (2000) confirmed that freshly harvested *etr 1-1* seeds did not germinate and observed that the germination of two other mutant *Arabidopsis thaliana* seeds with reduced sensitivity to ethylene (*ein 2-1* and *ein 2-45*) was also completely inhibited when the seeds were freshly harvested. However, in contrast to the observation of Bleecker et al. (1988), cold stratification for 4 days at 4°C, permitted total germination for all ethylene insensitive genotypes. Beaudoin et al. (2000)

also observed that ethylene insensitive seeds were more sensitive to exogenously applied ABA than WT *Arabidopsis thaliana* seeds. Germination of the ethylene insensitive seeds was almost completely inhibited in 0.3 μM ABA, a concentration that did not affect WT seed germination. Ghassemian et al. (2000) reported that the sensitivity of *etr Arabidopsis thaliana* seeds to exogenous ABA was age-dependent. Seeds 3 months post harvest were sensitive to 0.3 μM ABA, while those older than 3 months required ABA concentrations of 0.6 μM to inhibit their germination. Ghassemian et al. (2000) also reported that *ein* seeds accumulated higher quantities of ABA than WT *Arabidopsis* seeds. Beaudoin et al. (2000) observed that *Arabidopsis* seeds that had a phenotype resembling a constitutive exposure to ethylene (*ctr* seeds) had only slightly faster germination than WT *Arabidopsis* seeds. When strongly ethylene insensitive *Arabidopsis thaliana* plants (*etr 1-1*) were crossed to WT plants, the F1 seeds had slower germination, similar to homozygous *etr 1-1* seeds, but only when the maternal parent was ethylene insensitive.

The role of ethylene in *Petunia hybrida* germination appeared to be similar to that in *Arabidopsis*. Completely ethylene insensitive petunia seeds (*etr 1-1*) had lower germination than WT petunia seeds that could be rescued by application of GA (Clark, personal communication). Barry (2004) observed that the ability of *etr 1-1* petunia seeds to germinate was dependent on the stage of their development and duration of after-ripening. The same as in *Arabidopsis* requirement for the maternal parent to be ethylene insensitive was observed in *Petunia* seeds. Further, *etr 1-1* seeds were more sensitive to ABA than WT *Petunia* seeds (Barry, personal communication).

The role of ethylene in germination of other plant species, such as tomato, is more controversial. Siriwitayawan et al. (2003) reported that partially ethylene insensitive *Nr* tomato seeds from two different cultivars, 'Rutgers' and 'Ailsa Craig' completed germination 12 or, 24 hours, respectively, more rapidly than WT seeds from the same cultivars. Application of 5 mM 1-aminocyclopropane-1carboxylic acid (ACC) increased the speed of germination in WT tomato seeds but did not affect it in *Nr* seeds. Application of the ethylene action inhibitor, 2,5-norbornadiene (NBD) significantly reduced the speed of germination of both WT and *Nr* seeds, but decreased the percent germination in WT seeds only. Siriwitayawan et al. (2003) explained the lower than expected effect of the ethylene insensitivity on tomato germination with leakiness of the *Nr* mutation allowing some perception of ethylene. The authors also suggested that other than NR receptors could have physiological importance during tomato germination. Even though *Nr* seeds germinated faster than WT seeds, they were more adversely affected by accelerated aging or low temperature stress than WT seeds (Siriwitayawan et al., 2003). In order for the altered germination behaviors to be observed, the female parent had to be ethylene insensitive. It could be speculated that ethylene insensitivity might affect germination through affecting seed development.

Even though ethylene insensitive lettuce lines had been obtained in the cultivar 'Diana' (Salveit et al., 2003), their germination was not evaluated. The role of ethylene in lettuce germination had been examined only by using inhibitors of ethylene production or action. However, the studies involving the two types of inhibitors led to controversial results. Some authors reported that application of aminoethoxyvinylglycine (AVG) reduced lettuce germination (Abeles, 1986; Saini et al., 1989). Others (Nascimento, 1998)

did not observe inhibition of germination following AVG application, although ethylene production was inhibited. Possibly, by creating transgenic lettuce lines with reduced sensitivity to ethylene or altered ethylene biosynthesis, some of these discrepancies would be understood.

The objective of the present work was to examine how reduction of ethylene perception or alteration of ethylene production might affect germination of thermosensitive 'Dark Green Boston' and thermotolerant 'Everglades' seeds at different temperature conditions, optimal (20°C) and supraoptimal temperature (36°C), in continuous light or dark.

Material and Methods

Plant Material

Homozygous seed populations from the second (T2) generation were used (see chapter 5). For EVE ETR, the experiments were repeated with seeds from the third (T3) generation. Seeds from three ethylene insensitive (ETR) EVE (EVE ETR 2, 6 and 18) and ACC overexpressing (ACC) EVE lines (EVE ACC 1, 3, and 4), two DGB ETR lines (DGB ETR 6 and 14) and one DGB ACC line (DGB ACC 2) were used. Seeds harvested from individual plants were used for EVE ETR, EVE ACC (with the exception of EVE ACC 3) and DGB ACC lines. The seeds were combined for DGB ETR experiments because of a lack of sufficient number of seeds from the individual plants. The time period of seed development and maturation, temperatures during this period and duration of after-ripening prior to the start of the experiments are represented in Table 6-1. All the seeds were produced in greenhouses, at the Everglades Research and Education Center.

Table 6-1 Conditions during transgenic seed production

Genotype	Seed Maturation Period	Temperatures During Maturation (°C)	Duration of after-ripening
DGB ETRs	03/01/03-04/20/03	Min. 6.7 Max. 32.8	7 months
T2 EVE ETRs	07/01/01-07/20/01	Min. 19.4 Max. 33.3	11 months
T3 EVE ETRs	02/20/03-04/05/03	Min. 6.4 Max. 32.5	10 months
DGB ACCs	07/01/02-07/20/02	Min. 20.6 Max. 33.8	8 months
EVE ACCs	06/01/02-06/20/02	Min. 20.6 Max. 33.8	8 months

Germination Tests

The methodology for performing the germination tests is described in chapter 4.

Ethylene Determination

The methodology for measuring ethylene production during germination is described in chapter 4.

Experimental Design and Statistical Analysis

The experimental designs and the statistical analysis used were the same as those in chapter 4. Statistical analysis of the correlation between ethylene production and germination for all transgenic lines is represented in the Appendix.

Results and Discussion

Germination of Ethylene Insensitive DGB ETR Seeds

Germination of wild-type (WT) and ethylene insensitive (DGB ETR 6 and DGB ETR 14) seeds at temperatures ranging from 16ºC to 36ºC in light is represented in Table 6-2. DGB ETR 14-4 seeds were included in this experiment as additional control since they had neither resistance to glyphosate nor altered triple response. It was concluded that these seeds came from 'azygous' plants, lacking the T-DNA as a result of T-DNA segregation in the first generation. Germination of the ethylene insensitive lines (DGB ETR 6 and DGB ETR 14) was reduced by increasing the imbibition temperature from 20ºC to 24ºC, followed by second significant reduction when imbibition temperature

Table 6-2 Germination of WT and ethylene insensitive DGB seeds at temperatures from 16°C to 36°C in light

Temperature (°C)	Genotype			
	DGB ETR 6	DGB ETR 14	WT DGB	DGB ETR 14-4
16	96.7	100.0	100.0	100.0
20	100.0	100.0	100.00	100.0
24	68.3	65.0	100.0	98.3
28	41.7	5.0	18.3	33.3
32	10.0	8.3	10.0	3.3
36	10.0	1.7	6.7	3.3
LSD value			16.7	

Data were analyzed using ANOVA. Means were separated using Least Significant Difference (LSD) for the two way interaction, temperature and genotype.

was increased to 28ºC. DGB ETR 6 and DGB ETR 14 seeds had similar germination in light at 32ºC and 36ºC (Table 6-2). Germination of WT seeds (WT DGB ad DGB ETR 14-4) was reduced when imbibition temperature was increased from 24ºC to 28ºC, followed by second reduction with increase from 28ºC to 32ºC. Germination of both WT DGB and DGB ETR 14-4 seeds at 24ºC was higher than that of DGB ETR 6 and DGB ETR 14, 100 and 98% as compared to 63 and 65%, respectively. At 28ºC, DGB ETR 14-4 germination was higher than DGB ETR 14 germination but WT DGB germination was lower than DGB ETR 6 germination.

The rate of germination of the seeds from all lines at the different temperatures is represented in Figure 6-1. At 16ºC, the seeds from all lines not only had maximal germination but also germinated very uniformly. All seeds reached 100% germination by the 36th hour. No differences in germination rate between WT and ethylene insensitive lines were observed. At 20°C, even though all lines germinated, some of them (DGB ETR 14) had slower germination. At 24°C, the completion of germination was delayed in all seeds. DGB ETR 6 and DGB ETR 14 germination increased steadily, without single peak, starting from around 24 hours post imbibition and reaching 68 and 65%, respectively, at 120 hours. Germination of WT seeds started increasing earlier, at 20 hours, and reached maximum at 96 hours. At 28°, 32° and 36°C, germination of all lines was strongly reduced and nonuniform.

Germination of thermosensitive DGB seeds was reduced if their sensitivity to ethylene was reduced. Both DGB ETR 6 and DGB ETR 14 seeds had reduced germination at 24°C while WT seed germination was 100%. This indicated that production and perception of ethylene was required for germination even at this close to

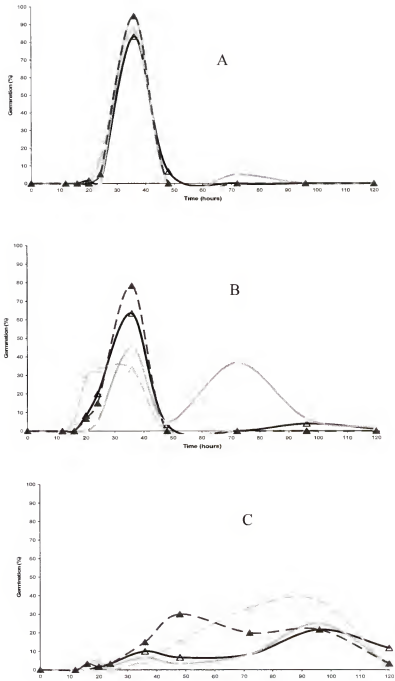


Figure 6-1 Speed of germination of WT DGB and DGB ETR seeds in light at temperatures from 16ºC to 36ºC. DGB ETR 6 is represented with solid lines, open triangles, DGB ETR 14 with solid lines, filled triangles, WT DGB with broken lines, open triangles and DGB ETR 14-4 with broken lines, filled triangles. A) 16°C. B) 20°C. C) 24°C. D) 28°C. E) 32°C. F) 36°C.

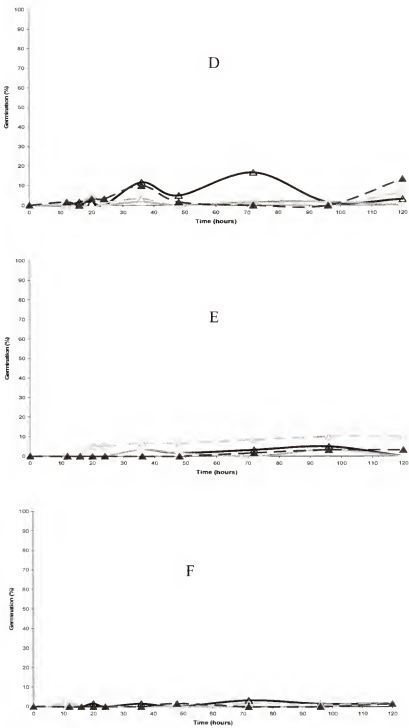


Figure 6-1. Continued.

the optimal temperature in DGB seeds. Reduction of ethylene perception by STS application also reduced DGB germination at optimal temperature (chapter 3). DGB ETR 14 seeds germinated slower than WT DGB seeds at 20°C and both DGB ETR 6 and DGB ETR 14 germinated slower than WT DGB seeds at 24°C. This indicated that reduced perception of ethylene resulted in increased time to germination as temperature increased. Siriwitayawan et al. (2003) reported similar delay in completion of germination in *Arabidopsis* ethylene insensitive seeds.

In order to better understand the role of ethylene during lettuce germination, DGB ETR 6 and DGB ETR 14 seeds were germinated at a range of temperatures (from 16°C to 36°C) in continuous dark (Table 6-3). No significant difference between WT DGB and DGB ETR germination at the different temperatures was observed. This indicated that both seeds with reduced and with normal (WT) ethylene perception responded to increasing imbibition temperatures in dark in a similar way. Germination of all DGB seeds was highest at 16°C. When imbibition temperature was increased to 20°C, DGB germination was significantly reduced, followed by second reduction with increase of temperature to 24°C and third to 28°C. Germination of DGB seeds at temperatures above 28°C was almost completely inhibited in dark (Table 6-3).

The rate of germination of WT and ethylene insensitive DGB seeds in continuous dark is represented in Figure 6-2. Even though at 16°C all seeds germinated at 100%, DGB ETR germination was slower than WT DGB germination. DGB ETR 6 and DGB ETR 14 reached maximal germination at 72 hours after the start of imbibition as compared to 48 hours for WT seeds. At 20°C, germination of all lines was strongly reduced and delayed.

Table 6-3 Germination of WT and ethylene insensitive DGB seeds at temperatures from 16°C to 36°C in constant dark

Temperature (°C)	Genotype				Means (Temperature)
	DGB ETR 6	DGB ETR 14	WT DGB	DGB ETR 14-4	
16	93.3	100.0	100.0	100.0	98.3
20	46.7	40.0	70.0	65.0	55.4
24	21.7	6.7	21.7	10.0	15.0
28	6.7	6.7	1.7	3.3	4.6
32	3.3	5.0	3.3	5.0	4.2
36	8.3	1.7	0.0	1.7	2.9
	LSD value				6.5

Data were analyzed using ANOVA. Means within the temperature were separated using Least Significant Difference (LSD).

When imbibition of WT and ethylene insensitive DGB seeds was conducted in continuous dark, their germination was reduced at lower temperatures than it was in continuous light. It is possible that the reduced ethylene production in dark (chapter 4) leads to reduction of DGB germination at lower temperatures as compared to light germination. It appears that DGB seeds become extremely thermosensitive in dark at temperatures equal or higher than 20°C. There were no differences in the germination response of WT DGB and DGB ETR seeds to increasing temperatures in dark. A possible explanation for this may be that the ability of DGB seeds to produce ethylene may be reduced so much in dark that it will lead to reduced germination of both DGB seeds with normal and reduced ethylene perception.

In order to correlate ethylene production and germination in DGB ETR lines, the amounts of ethylene produced by WT and ethylene insensitive DGB seeds in light and dark were measured (Table 6-4). At 20°C, in both light and dark, all DGB seeds germinated at 100%. The two ethylene insensitive lines (DGB ETR 6 and DGB ETR 14) and WT DGB produced similar amounts of ethylene in both light and dark at 20°C (Table 6-4). Ethylene production by all seeds was higher in light than in dark.

Ethylene production and germination were measured during imbibition of DGB ETR lines and WT DGB at temperatures above 27°C (Table 6-5). In light, 36°C was used as supraoptimal temperature and in dark 28°C. The means from the different lines were analyzed separately for each temperature. At 36°C, in light, DGB ETR seeds and WT DGB seeds produced similar amounts of ethylene and had similar, low germination (Table 6-5). At 28°C, in dark, WT DGB seeds had significantly greater germination than

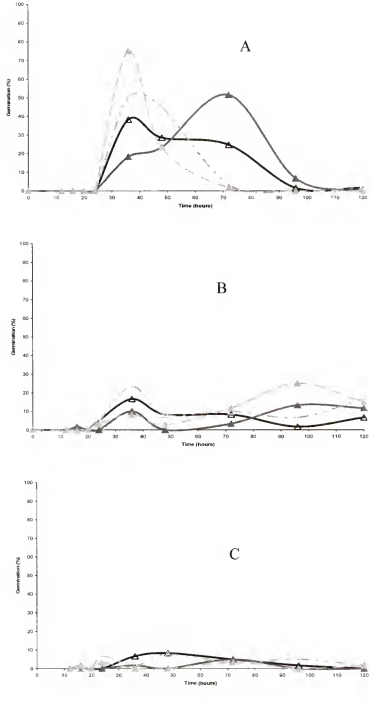
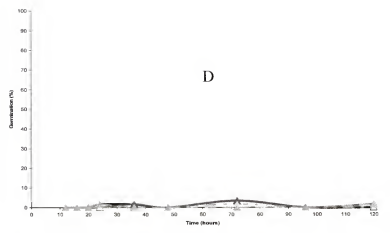
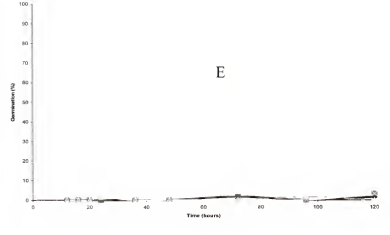


Figure 6-2 Speed of germination of WT DGB and DGB ETR seeds in dark at temperatures from 16°C to 36°C. DGB ETR 6 is represented with solid line, open triangle, DGB ETR 14 with solid line, filled triangles, WT DGB with broken line, open triangles, and DGB ETR 14-4 with broken line, filled triangles. A) 16°C. B) 20°C. C) 24°C. D) 28°C. E) 32°C. F) 36°C.





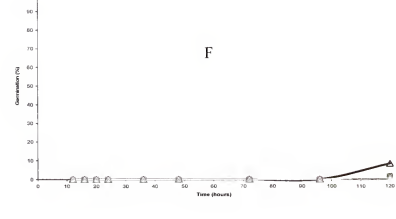


Figure 6-2 Continued.

Table 6-4 Ethylene production (nl/g/h) of WT and DGB ETR seeds at 20°C in light and dark

Genotype	Light	Dark
DGB ETR 6	28.96	9.83
DGB ETR 14	26.76	10.17
WT DGB	28.39	8.14
Means	28.04	9.38**
(Light conditions)		

The germination percentages and the average ethylene production were analyzed using ANOVA. The average ethylene production was calculated using ethylene production values from 7 time points during the course of 48 hours of imbibition. The percent germination after 48 hours was used for analysis. Means within light conditions were separated using Least Significant Difference (LSD).

Table 6-5 Germination and ethylene production of WT and DGB ETR seeds at 36°C in light and 28°C in dark

Genotype	36°C in Light		28°C in Dark	
	Germination (%)	Ethylene (nl/g/h)	Germination (%)	Ethylene (nl/g/h)
DGB ETR 6	11.1	3.47	17.8	4.78
DGB ETR 14	4.5	1.23	8.3	1.63
WT DGB	12.2	7.76	62.2	7.11
LSD Values	NS		19.4	1.8

The germination percentages and the average ethylene production were analyzed using ANOVA. The average ethylene production was calculated using ethylene production values from 7 time points during the 48 hours of imbibition. The percent germination at 48 hours was considered final and used for analysis. Means within column were separated using Least Significant Difference (LSD).

DGB ETR 6 and DGB ETR 14 seeds. WT DGB seeds also produced significantly more ethylene than the two ethylene insensitive DGB lines (Table 6-5).

Germination and ethylene production of DGB ETR 14 and WT DGB seeds during 48 hours of imbibition in light are represented in Figure 6-3. At 20°C, DGB ETR 14 seeds started to produce ethylene at 12 hours after the start of imbibition. The peak of ethylene production was at 36 hours. Also at 36 hours, DGB ETR 14 seeds reached almost 100% germination. At 36°C, DGB ETR 14 ethylene production was low, with a peak at 36 hours (Figure 6-3). Germination of the ethylene insensitive seeds was also low, with a peak at 48 hours. At 20°C, ethylene production in WT DGB seeds also started at 12 hours but reached peak at 24 hours (as compared to 36 hours for DGB ETR 14 seeds). WT DGB germination reached almost 100% also at 24 hours. At 36°C, WT DGB seeds produced more ethylene than DGB ETR seeds and also had earlier peak of ethylene production (at 20 hours as compared to 36 hours for DGB ETR 14). Both ethylene production and germination were delayed in DGB ETR 14 seeds as compared to WT DGB seeds at 20°C.

Germination and ethylene production by DGB ETR 14 and WT DGB seeds in dark over 48 hours of imbibition are represented in Figure 6-4. Germination of DGB ETR 14 seeds at 20°C increased throughout the whole imbibition and reached close to 100% at 48 hours. DGB ETR 14 seeds also produced most ethylene at 48 hours at 20°C. At 28°C, ethylene production of DGB ETR 14 seeds was low. However, it increased during imbibition and both ethylene production and germination were greatest at 48 hours. At 20°C, ethylene production of WT DGB seeds increased sooner than ethylene production

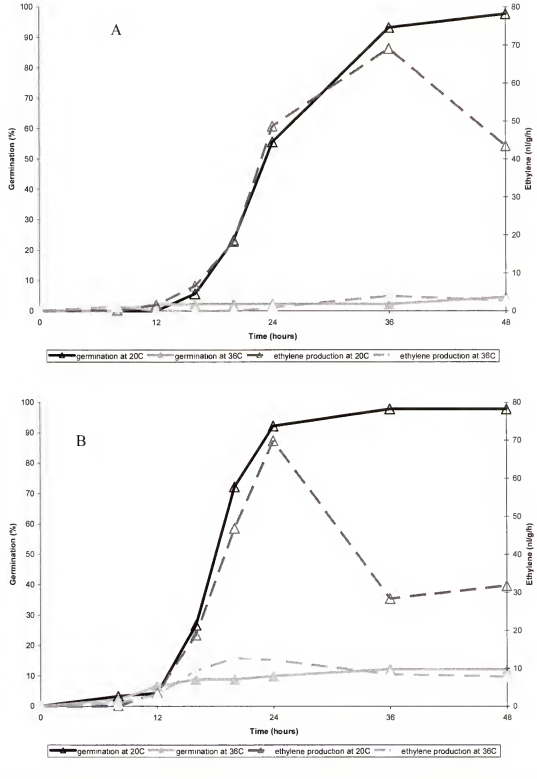


Figure 6-3 Germination (solid lines) and ethylene production (broken lines) of DGB ETR 14 and wt DGB seeds at 20°C (open triangles) and 36°C (filled triangles) in light. Vertical bars indicate standard errors. A) DGB ETR 14. B) WT DGB.

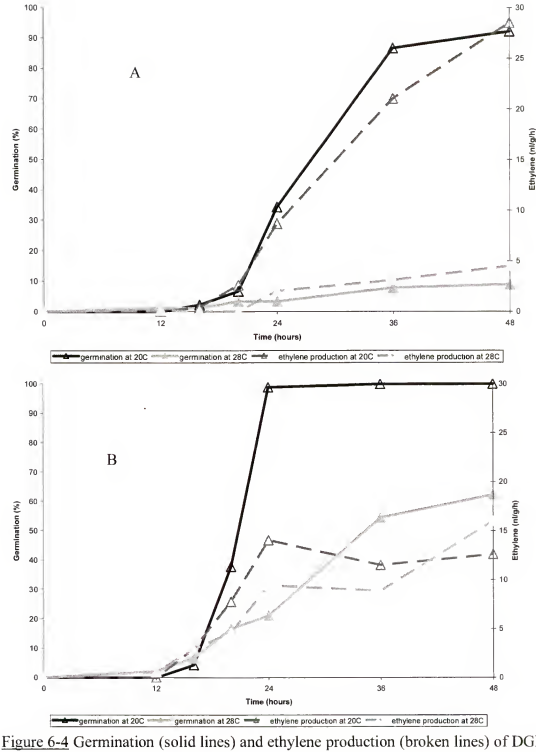


Figure 6-4 Germination (solid lines) and ethylene production (broken lines) of DGB ETR 14 and wt DGB seeds imbibed for 48 hours at 20°C (open triangles) and 28°C (filled triangles) in dark. A) DGB ETR 14. B) WT DGB.

of DGB ETR 14 seeds, reaching maximum at 24 hours after the start of imbibition. At the same 24-hr period, WT DGB germination reached 100%. Ethylene production of WT DGB seeds at 28°C was greater than that of DGB ETR 14 seeds. Both ethylene production and germination of WT DGB seeds were greatest at 48 hours. Similar to the imbibition in light, at 20°C in dark, WT DGB seeds both produced more ethylene and germinated faster than DGB ETR 14 seeds. This delay in ethylene production might be related to the reduced sensitivity to ethylene.

Measurements of ethylene production during germination in light provided some understanding about the way reduced perception of ethylene might affect DGB germination. At 20°C, although all DGB ETR seeds and WT DGB seeds produced similar amounts of ethylene, the rate of ethylene produced over time was lower in DGB ETR seeds as compared to WT DGB seeds. This delay may be attributed to the reduced ethylene perception. How reduced ethylene perception may lead to delayed ethylene production is unknown. It has been reported that ethylene production is auto stimulated by ethylene itself in germinating pea seeds (Petruzzelli et al., 2000). It may be possible that the lower ability to perceive ethylene results in delayed auto-stimulation of ethylene production.

The importance of unaltered ethylene perception for lettuce germination, especially at supraoptimal temperatures, was confirmed by ethylene determination in dark. Although the amount of ethylene produced at 20°C in dark was reduced to half that in light, all DGB lines germinated close to 100%. This indicated that thermosensitive DGB seeds probably produced more ethylene at optimal temperature in light than was required for germination. However, at optimal temperature in dark, a reduction in the

ability to perceive ethylene affected the timing of ethylene production, and, consequently, germination in DGB.

At supraoptimal temperatures both ethylene production and germination of all lines were reduced. However, both the amount of ethylene produced and germination were significantly lower for the DGB ETR lines indicating that under these experimental conditions reduced sensitivity to ethylene might have resulted in reduced germination. Ethylene production by DGB ETR 6 seeds was reduced in half as compared to ethylene production by WT DGB seeds while DGB ETR 6 germination was reduced around three times. Possibly, at 28°C in dark, DGB ETR 6 seeds may produce high amount of ethylene but not in enough quantity to achieve the level of germination in WT DGB. WT DGB seeds produced a similar amount of ethylene (7 nl/g/h) at 28°C in dark to that produced at 20°C in dark (8 nl/g/h). However, at 28°C, WT seeds germinated only at 60% of the level of germination at 20°C. This further supports the hypothesis that germination at supraoptimal temperatures requires higher amounts of ethylene as compared to germination at optimal temperatures.

In conclusion, reduction of ethylene perception in thermosensitive DGB seeds resulted in a reduction in germination at both optimal and supraoptimal temperatures. At optimal temperatures, ethylene insensitive DGB seeds had a delay in ethylene production that resulted in delayed and non-uniform germination. At supraoptimal temperatures, ethylene insensitive seeds produced less ethylene and consequently had lower germination than seeds with normal ethylene perception.

Germination of Second Generation (T2) Ethylene Insensitive EVE ETR Seeds

Transgenic lines with reduced perception of ethylene were also obtained from the thermotolerant genotype 'Everglades' (EVE). Germination of homozygous seed populations from two plants from two transgenic lines (EVE ETR 6 and EVE ETR 18) and one plant from the line EVE ETR 2 in both continuous light and dark was tested. Two seed populations with wild-type sensitivity to ethylene were included in the experiments. The first population that was designated WT EVE came from a non-transformed EVE plant while the other population (EVE ETR 2-12) came from a plant, which was not resistant to glyphosate and with WT triple response and was considered 'azygous', lacking the T-DNA.

At 20ºC in light, all EVE ETR seeds and WT EVE seeds germinated at 100% (Table 6-6). Increase in the temperature of imbibition from 20ºC to 36ºC did not reduce germination in any line.

When imbibition was conducted in continuous dark, germination of all ETR and WT seeds was again 100% at 20ºC (Table 6-6). However, at 36ºC in dark, germination of all EVE ETR lines was significantly reduced as compared to both WT EVE and EVE ETR 2-12 germination. From all ETR lines, germination of EVE ETR 6-6 was the lowest (10%) and that of EVE ETR 18-5 the highest (27 %). Germination of WT EVE and EVE ETR 2-12 seeds was not reduced at 36ºC.

The rate of germination of WT EVE and EVE ETR seeds was also accounted in order to observe whether the reduced ethylene perception affected uniformity and speed of germination of the thermotolerant EVE seeds. At 20ºC in light, all ETR and WT EVE lines had rapid germination, without differences between the seeds with different ability

Table 6-6 Germination of WT and ethylene insensitive EVE seeds at 20°C and 36°C

Genotype	Light		Dark	
	Temperature		Temperature	
	20°C	36°C	20°C	36°C
EVE ETR 2-5	100.0	96.7	100.0	26.7
EVE ETR 6-6	100.0	96.7	100.0	10.0
EVE ETR 6-7	100.0	100.0	100.0	13.3
EVE ETR 18-1	100.0	100.0	100.0	16.7
EVE ETR 18-5	100.0	100.0	100.0	26.7
WT EVE	100.0	100.0	100.0	100.0
EVE ETR 2-12	100.0	100.0	96.7	96.7
LSD Value	NS		8.2	

Data were analyzed using ANOVA. Means were separated using Least Significant Difference (LSD) for the two way interaction, temperature and genotype.

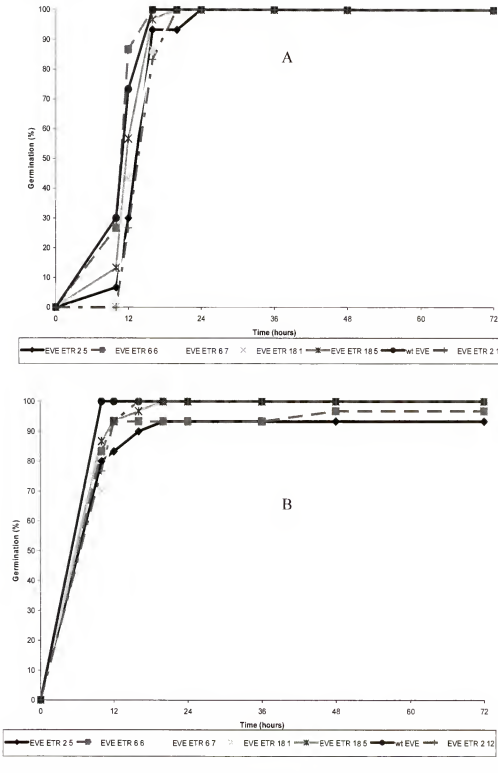
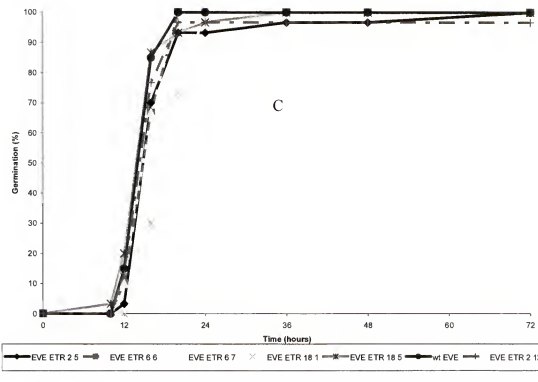


Figure 6-5 Speed of germination of WT and ethylene insensitive EVE seeds at 20ºC and 36ºC, in either light or dark. A) 20ºC in light. B) 36ºC in light. C) 20ºC in dark. D) 36ºC in dark.



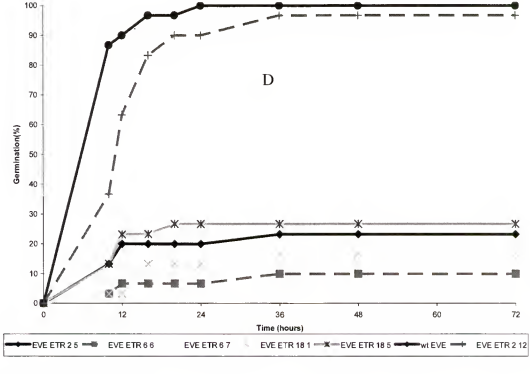


Figure 6-5. Continued.

to perceive ethylene (Figure 6-5). Germination of all seeds started at 10 hours after the start of imbibition and was complete by 16 hours. At 36ºC in light, most seeds had almost complete germination at 10 hours (Figure 6-5). Germination of WT EVE seeds was 100%. When imbibition at 20ºC was conducted in dark, germination in all lines was delayed as compared to germination in light. First germinated seeds were detected in all lines at 12 hours and germination was complete by 20 hours (Figure 6-5).
There were no differences in the speed of germination between WT seeds and those with reduced ethylene perception. The only exception was line EVE ETR 18-1 that germinated slower than all other lines. At 36ºC in dark, germination of WT EVE seeds was again very high at 10 hours after the start of imbibition and reached 100% by 16 hours. Germination of EVE ETR 2-12 seeds was low at 10 hours but reached almost 100% at 36 hours. Germination of all EVE ETR lines was very low at 36ºC in dark. It started at 10 hours and did not increase past 20 hours.

Germination behavior of thermotolerant WT and ethylene insensitive EVE seeds was different from that of thermosensitive DGB seeds. WT EVE seeds germinated at 100% at both optimal (20ºC) and supraoptimal (36ºC) temperatures in either dark or light, while germination of WT DGB at 36°C was poor. At 20ºC, WT EVE seeds started to germinate much earlier than WT DGB seeds. Nascimento et al. (2000) reported that EVE seeds produced 10 times more ethylene during germination than DGB seeds. This may account why EVE are thermotolerant. The only experimental condition at which EVE ETR germination was significantly reduced as compared to seeds with unaltered ethylene perception was at 36ºC in dark The high germination of EVE ETR seeds at

36°C in light may be explained by high ethylene production by the EVE ETR seeds in light.

In order to examine the effect of reduced sensitivity to ethylene on germination of thermotolerant EVE seeds under different experimental conditions, germination of WT and EVE ETR seeds in the ethylene action inhibitor, silver thiosulfate (STS), and/or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) was examined in light and dark. In light, there was no difference in germination of ethylene insensitive EVE lines and WT EVE in STS, ACC, or combinations of both (Table 6-7). Seeds from the three ethylene insensitive EVE lines (EVE ETR 2-5, EVE ETR 6-6, and EVE ETR 18-1) and WT EVE seeds germinated 100% in water, regardless of imbibition temperature. Seeds from all lines also had 100% germination in ACC at both imbibition temperatures. Application of STS did not affect either EVE ETR or WT EVE germination at 20°C but significantly reduced germination of all seeds at 36°C. When ACC was applied simultaneously with STS, all seeds germinated close to 100% at 20°C but had intermediate (between that in STS and ACC alone) germination at 36°C.

In order to further examine the possible effects of reduced ethylene perception on germination of thermotolerant EVE seeds in light, the rate of germination of these seeds was recorded (Figure 6-6). Germination in water at both 20°C and 36°C in light was very rapid for both ETR and WT EVE lines. Although application of STS did not reduce germination of any line at 20°C, it delayed the onset of radicle protrusion. The germination delay was more prominent for EVE ETR lines. Germination of all seeds was low in 20 mM STS at 36°C. It started at the same time as did germination in water but it was completely inhibited past 15 hours of imbibition. Imbibition in 10 mM ACC at 20°C

Table 6-7 Germination of WT and EVE ETR seeds in water, 20 mM STS, 10 mM ACC and 20 mM STS + 10 mM ACC at 20ºC and 36ºC in light

Genotype	Temperature/Solution							
	20ºC				36ºC			
	Water	STS	ACC	STS + ACC	Water	STS	ACC	STS + ACC
EVE ETR 2-5	100.0	100.0	96.7	93.3	100.0	33.3	100.0	53.3
EVE ETR 6-6	100.0	93.3	93.3	96.7	100.0	23.3	100.0	50.0
EVE ETR 18-1	100.0	90.0	90.0	96.7	100.0	23.3	100.0	76.7
WT EVE	100.0	100.0	100.0	100.0	100.0	33.3	100.0	80.0
Means (Solutions X Temperature)	100.0	95.8	99.2	96.7	100.0	28.3	100.0	65.0
LSD Value	8.6							

Data were analyzed using ANOVA. The abbreviation STS stands for silver thiosulfate and ACC for 1-aminocyclopropane-1-carboxylic acid. Means for the solutions at the different temperatures were separated using Least Significant Difference (LSD).

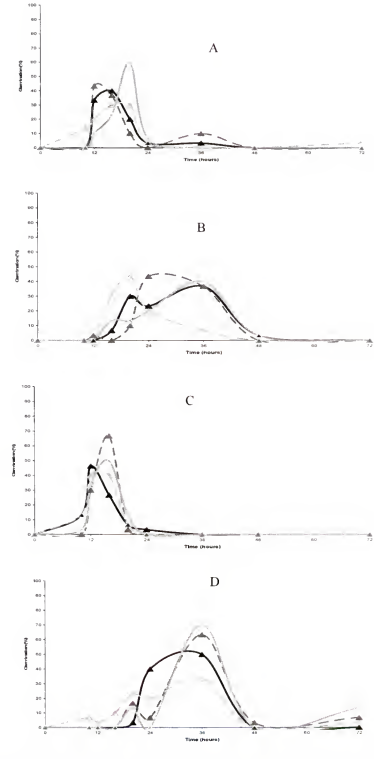


Figure 6-6 Speed of germination of wt EVE and EVE ETR seeds at 20°C (A-D) and 36°C (E-H) in light. EVE ETR 2-5: solid line, filled triangles; EVE ETR 6-6: broken line, filled triangles; EVE ETR 18-1: solid line, open triangles and wt EVE with broken line, open triangles. E) Water. B, F) STS. C,G) ACC. D,H) ACC + STS.

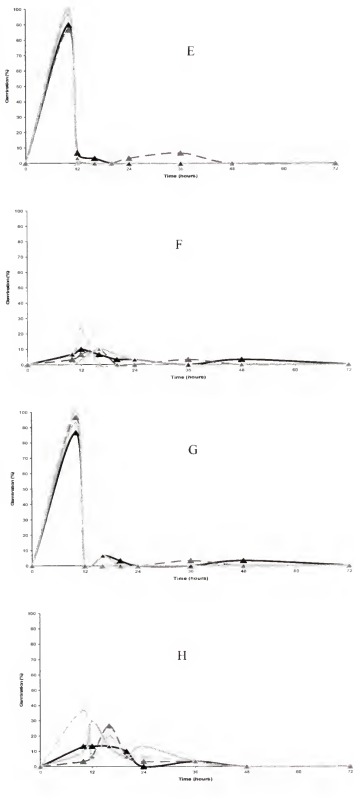


Figure 6-6. Continued.

in light led to slightly more rapid germination of all seeds as compared to water alone. All lines completed germination by 16 hours of imbibition as compared to 20 hours for water. At supraoptimal temperature, the application of ACC did not have any effect on WT or EVE ETR germination rate. Simultaneous application of ACC and STS at 20°C did not increase either the speed of germination or the final percent germination as compared to imbibition in STS alone. In both solutions, seeds from all genotypes started to germinate around 20 hours after the start of imbibition and germination was completed by 36 hours. At 36°C, simultaneous application of ACC to STS increased the percent germination over STS alone in all lines. However, ACC did not affect the timing of germination. In all genotypes, germination was complete by 24 hours.

There were no interactions between the reduced ethylene perception and the effect of STS or ACC in light during germination of WT EVE and EVE ETR seeds. The observation that germination of all EVE ETR lines and WT EVE seeds was 100% in both water and ACC in either optimal or supraoptimal temperature indicated that EVE seeds might produce enough ethylene at both temperature conditions to ensure germination. Application of ACC did not increase the speed of germination of either WT or ETR EVE seeds indicating that the production of endogenous ethylene was most probably sufficient. ACC had been reported to promote germination of lettuce and other crops, such as tomato and sweet corn under stress, but to have little effect on their germination under optimal conditions (Khan, 1994b).

Reduced ethylene perception did not affect EVE germination at 36°C in light. This could be explained by either low requirement for ethylene by this genotype for germination at the supraoptimal temperature in light or production of higher amounts of

ethylene, capable of overcoming the reduced ethylene perception, by the ETR lines than WT EVE. The second hypothesis seems more probable. Siriwitayawan et al. (2003) reported that application of an ethylene action inhibitor, 2,5-norbornadiene reduced the rate of germination in *Nr* tomato seeds. Thus, it was thought that EVE ETR germination would be more strongly affected by STS than WT EVE germination, at least at the supraoptimal temperature. However, the response of EVE ETR and WT EVE seeds to STS at either temperature in light was similar (Table 6-7). The reason for this is unknown.

STS appeared to affect EVE germination in a different way at optimal and supraoptimal temperatures. At 20ºC, both WT and EVE ETR seeds germinated much slower in STS than in water or ACC. It is possible that this may be the result of a delay in ethylene production. Delayed ethylene production in 20 mM STS was reported by Nascimento et al. (2004a) in EVE seeds. At 36ºC in STS, EVE seeds germinated up to 33% and the germination did not increase past 24 hours of imbibition. The exact reason for the inability of EVE seeds to germinate past 24 hours at 36ºC in STS is unknown. It is possible that since EVE germination proceeds quickly at 36ºC in light, there are no more receptors synthesized past 24 hours. Application of ACC simultaneously with STS increased germination of all EVE seeds at 36ºC in light but did not increase the speed of germination at either temperature. This indicated that providing more ACC for production of more ethylene can not alone increase the speed of germination.

When WT and EVE ETR seeds were imbibed in dark, their germination was 100% in water at 20ºC (Table 6-8). At 36ºC, germination of all seeds was significantly reduced. Germination of the EVE ETR lines was lower than that of WT EVE seeds.

Table 6-8 Germination of WT and EVE ETR seeds in water, 20 mM STS, 10 mM ACC and ACC + STS at 20ºC and 36ºC in dark

Genotype	Solution/Temperature							
	H2O		STS		ACC		STS + ACC	
	20ºC	36ºC	20ºC	36ºC	20ºC	36ºC	20ºC	36ºC
EVE ETR 2-19	100.0	15.5	80.0	0.0	100.0	86.7	97.8	2.7
EVE ETR 6-7	100.0	24.5	91.1	0.0	100.0	80.0	97.8	0.0
EVE ETR 18-1	100.0	22.2	66.7	0.0	100.0	97.8	100.0	13.3
WT EVE	100.0	80.0	84.5	0.0	100.0	100.0	100.0	13.3
LSD Value	9.1							

Data were analyzed using ANOVA. The abbreviation STS stands for silver thiosulfate and ACC for 1-aminocyclopropane-1-carboxylic acid. Means were separated using the Least Significant Difference (LSD) for the three way interaction genotype X solution X temperature.

Application of ACC at 36ºC significantly increased germination of all seeds. Germination of WT EVE seeds was significantly greater than that of EVE ETR 2-19 and EVE ETR 6-7 seeds in ACC (Table 6-8). Application of STS significantly reduced EVE ETR 2-19 and EVE ETR 18-1 germination at 20ºC and completely inhibited germination of all lines at 36ºC. When ACC was applied simultaneously with STS, the effect of STS was completely reversed at 20ºC for all lines. At 36ºC, addition of ACC to STS improved slightly germination of EVE ETR 18-1 and WT EVE as compared to their germination in STS alone (Table 6-8).

Imbibition in dark did not affect the speed of germination of WT or ETR lines at either temperature as compared to the speed of germination in light. At both temperatures, EVE seeds completed their germination up to 16 hours after the start of imbibition, similar to seeds imbibed in light. At 20ºC, the speed of germination of all seeds was similar in ACC and in water (Figure 6-7). Imbibition in ACC increased germination at 36ºC without affecting its speed. Simultaneous application of ACC and STS did not increase either the percent germinated seeds in any of the lines at 20ºC or the speed of germination. At 36ºC, the speed of germination was also not increased by ACC as compared to STS alone.

Measurements of germination of thermotolerant EVE seeds with WT or reduced ethylene perception in light and dark, in presence of ACC, STS, or combinations of both confirmed that reduced EVE ETR germination in water at supraoptimal temperature is the result of reduced ethylene perception and provided new evidence for the importance of ethylene for lettuce germination. The observation that application of ACC significantly improved germination of all EVE ETR lines suggested that these lines were only partially

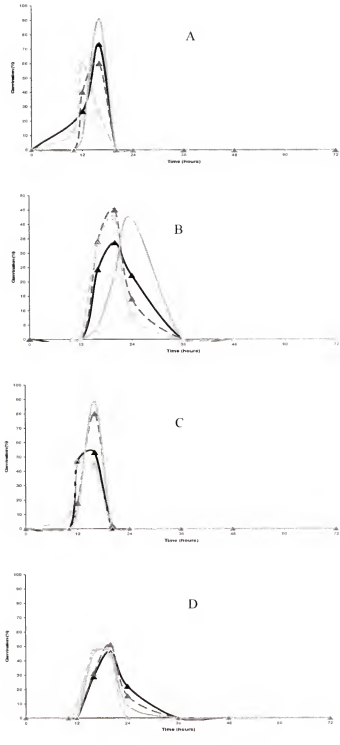


Figure 6-7 Speed of germination of WT and EVE ETR seeds at 20°C (A-D) and 36°C (E-H) in dark. EVE ETR 2-5: solid line, filled triangles; EVE ETR 6-6: broken line, filled triangles; EVE ETR 18-1: solid line, open triangles and WT EVE: broken line, open triangles. E) Water. B, F) STS. C, G) ACC. D, H) STS + ACC.

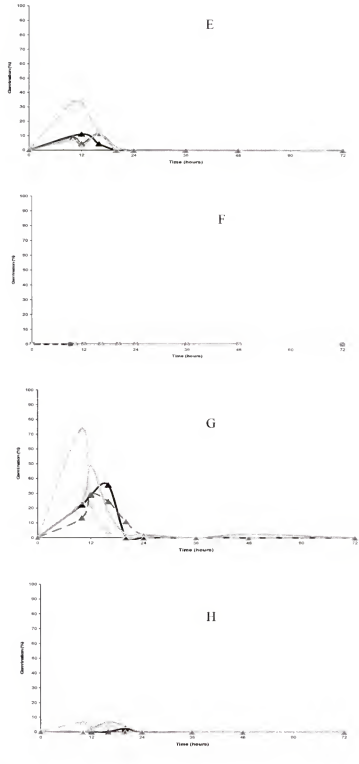


Figure 6-7. Continued.

ethylene insensitive. This conclusion was supported by the triple response phenotype of the ethylene insensitive lines which suggested retention of some sensitivity to ethylene (chapter 5). Siriwitayawan et al. (2003) reported that partially ethylene insensitive tomato *Nr* seeds also responded to exogenous ACC and exhibited reduction in the time required for completion of germination. The same authors also observed that some of the ethylene insensitive mutant *Arabidopsis* seeds were able to respond to exogenous ACC. They hypothesized that whether the ethylene insensitive seeds would respond to ACC depended on the strength of their phenotype. It is known that ethylene insensitivity conferred by *etr* 1-1 is stronger than ethylene insensitive phenotypes conferred by mutations in other members of the ethylene receptor family or other ethylene signaling pathway components. Since EVE ETR seeds were only partially ethylene insensitive, their germination behavior was the result of a balance between ethylene perception and ethylene production. It is possible that ACC stimulates EVE ETR germination at 36ºC in dark by supplying sufficient quantity of the immediate ethylene precursor and thus stimulating ethylene production, and, consequently, their germination. Nascimento et al. (2004a) reported that both EVE and DGB seeds produced from 5 to 10 times more ethylene when imbibed in 10 mM ACC than in water.

Application of 20 mM STS at 20ºC had the same effect on EVE ETR germination (with the exception of EVE ETR 18-1) as on WT EVE germination. This observation contradicted to the results obtained by Siriwitayawan et al. (2003). The authors reported stronger effect of 2,5-norbornadiene on speed of germination of *Nr* seeds as compared to WT tomato seeds. This discrepancy can be explained by the use of two different types of ethylene action inhibitors. 2,5-Norbornadiene is an ethylene inhibitor that competes with ethylene for binding to the receptors. It is possible that since etr 1-1 mutant receptors can

not bind to ethylene (Bleecker et al., 1998) they may also be unable to bind 2, 5-norbornadiene. This means that in ethylene insensitive tissues there might be less functional receptors to compete for. In contrast, STS has been hypothesized to function as an ethylene inhibitor by displacing copper cations, which are normally located inside the receptors, with silver cations (Rodriguez et al., 1999). It may be possible that STS does not have any preference for replacing the copper in functional (able to bind ethylene) or dysfunctional (unable to bind ethylene) receptors.

Ethylene production by the three EVE ETR lines and WT EVE seeds was measured during germination in light (Table 6-9). The interaction between genotypes and temperatures was nonsignificant for both germination and ethylene production. All seeds germinated at 100% or close to 100% at either temperature. Average ethylene production for 20ºC and 36ºC was significantly higher in EVE ETR 2-20 and EVE ETR 6-7 seeds than in WT EVE seeds (Table 6-9). Average ethylene production at 36ºC was significantly higher that that at 20ºC.

In order to understand the reason for the different germination of EVE ETR seeds in light versus dark, ethylene production during imbibition in dark was measured for the three EVE ETR and the WT EVE seeds (Table 6-10). The two way interaction between genotypes and temperatures was significant for both germination and ethylene production. Both WT and EVE ETR seeds germinated at 100% at 20ºC. At 36ºC, germination of EVE ETR 2-20 and EVE ETR 6-7 was significantly reduced as compared to germination of WT EVE. At 20ºC, EVE ETR 6-7 produced more ethylene than WT EVE (Table 6-10). At 36ºC, ethylene production of WT EVE was greater than at 20°C and significantly greater than that of EVE ETR 2-20 and EVE ETR 18-1.

Table 6-9 Ethylene production of WT and EVE ETR seeds at 20°C and 36°C in light

Genotype	20°C	36°C	Means
	Ethylene (nl/g/h)		(Genotype)
EVE ETR 2-20	79.05	125.84	102.44
EVE ETR 6-7	93.75	125.60	109.67
EVE ETR 18-1	65.07	85.32	75.20
WT EVE	45.81	69.60	57.7
Means (Temperature)	70.92	101.59**	
LSD Value			18.0

Data were analyzed using ANOVA. The values for ethylene production were calculated by averaging ethylene production over 48 hours of seed incubation. Ethylene was evaluated very three hours. Means within temperature and genotype were separated using Least Significant Difference (LSD) values.

Table 6-10 Germination and ethylene production of WT and ethylene insensitive EVE seeds at 20°C and 36° in dark

Genotype	20°C		36°C	
	Germination (%)	Ethylene (nl/g/h)	Germination (%)	Ethylene (nl/g/h)
EVE ETR 2-20	100.0	20.72	63.3	19.54
EVE ETR 6-7	100.0	21.91	76.7	26.82
EVE ETR 18-1	100.0	13.40	100.0	22.63
WT EVE	100.0	13.14	100.0	31.53
LSD value	12.1	8.1	12.1	8.1

Data were analyzed using ANOVA. The means for the two way interaction genotype X temperature were separate using Least Significant Difference (LSD) values. The values for ethylene production were calculated by averaging ethylene production over 48 hours of seed imbibition. Ethylene was evaluated every three hours.

The time course of ethylene production and germination for EVE ETR 2-20 and WT EVE seeds in light is represented in Figure 6-8. The trends were similar for the two other EVE ETR lines. At 20°C, EVE ETR 2-20 germination increased rapidly, from 0% at 8 hours after the start of imbibition to 98% at 16 hours. Ethylene production at 20°C increased from 0 nl/g/h at 8 hours to 14 nl/g/h at 12 hours and reached peak at 20 hours (141 nl/g/h) (Figure 6-8). EVE ETR 2-20 germination began at 8 hours at 36°C and was complete at 16 hours. At 36°C, ethylene production of EVE ETR 2-20 seeds reached peak at the same time as did ethylene production at 20°C (Figure 6-8).

In light, at 20°C, WT EVE germination started at the same time as did EVE ETR 2-20 germination and also reached 100% at the same time (Figure 6-8). Ethylene production from WT EVE seeds initiated at 12 hours and peaked at 20 hours. At 36°C, germination of WT EVE seeds was complete at 12 hours of imbibition (Figure 6-8). Ethylene production of WT EVE seeds started 4 hours earlier at 36°C than at 20°C.

The time course of ethylene production and germination in dark for both EVE ETR 2-20 and WT EVE was different from that in light (Figure 6-9). EVE ETR 2-20 germination at 20°C increased more slowly than in light, reaching 100% at 20 hours of imbibition (as compared to 16 hours in light). The amount of ethylene produced by EVE ETR 2-20 seeds at 20°C was lower than that produced in light. There was no peak of ethylene production at 20°C or 36°C in dark. At 36°C in dark, EVE ETR germination was slow, reaching 63% at 48 hours (Figure 6-9). The amount of ethylene produced by EVE ETR 2-20 seeds at 36°C in dark was lower than that in light and comparable to that produced at 20°C in dark.

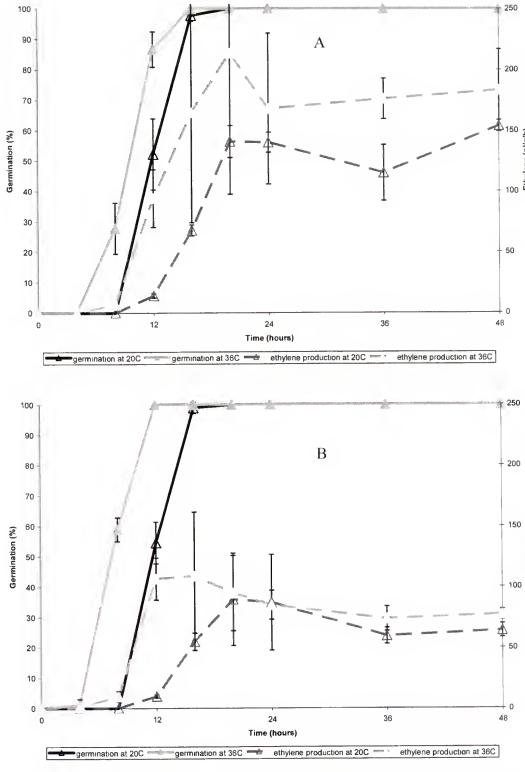


Figure 6-8 Germination (solid lines) and ethylene production (broken lines) of EVE ETR 2-20 and WT EVE seeds imbibed at 20°C (open triangles) and 36°C (filled triangles) in light. Vertical bars represent standard errors. A) EVE ETR 2-20. B) WT EVE.

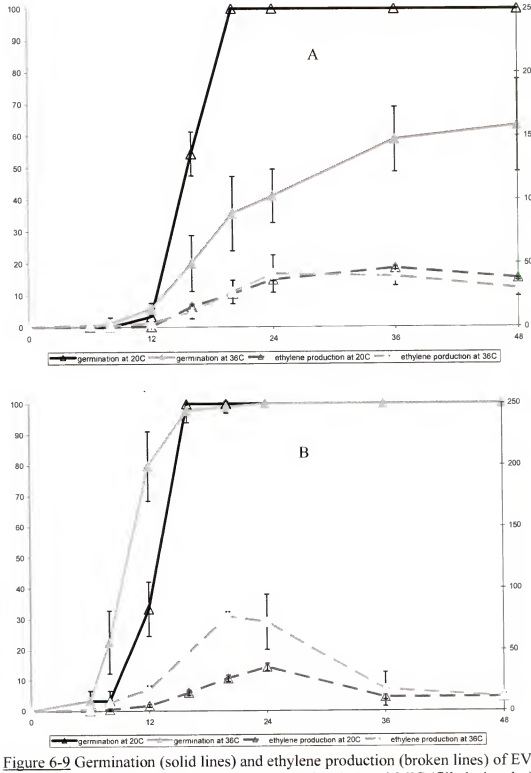


Figure 6-9 Germination (solid lines) and ethylene production (broken lines) of EVE ETR 2-20 and WT EVE seeds imbibed at 20ºC (open triangles) and 36ºC (filled triangles) in dark. Vertical bars represent standard errors. A) EVE ETR 2-20. B) WT EVE.

The rate of germination of WT EVE seeds at 20ºC in dark was the same as that of WT EVE seeds in light and higher than that of EVE ETR 2-20 seeds in dark (Figure 6-9). However, the peak of ethylene production was later in dark than in light (24 hours as compared to 20) and the greatest amount of ethylene produced was lower in dark than in light. Germination of WT EVE seeds at 36ºC was not different in dark from that in light, although germination was slower (peak at 16 hours as compared to 12 hours in light).

Measurements of ethylene production during germination of WT EVE and EVE ETR seeds provided important information about the complexity of germination regulation by ethylene perception and production. In light, EVE ETR 2-20 and EVE ETR 6-7 seeds produced more ethylene than WT EVE seeds indicating that ethylene insensitive seeds are capable of producing more ethylene than WT EVE seeds. A similar phenomenon was observed in *Nr* tomato seeds (Siriwitayawan et al., 2003). Ethylene insensitive 'Rutgers' tomato seeds produced 3.2 nl/g/h ethylene as compared to 0.6 nl/g/h for WT 'Rutgers' seeds, while 'Ailsa Craig' *Nr* seeds produced 21.6 nl/g/h as compared to 1.1 for WT seeds. Overproduction of ethylene was also reported for *etr* 1-1 *Arabidopsis* seeds, and *ein*1 and *ein*2 *Arabidopsis* etiolated seedlings and leaves (Guzman and Ecker, 1990; Siriwitayawan et al., 2003).

EVE ETR seeds also had different patterns of ethylene production throughout the period of imbibition. In WT EVE seeds, ethylene production peaked at the time of radicle protrusion or slightly after, indicating that completion of germination was associated with maximal ethylene production. Nascimento (1998) hypothesized that ethylene was produced throughout the whole imbibition but was 'trapped' inside the seeds and released at the time of radicle protrusion. However, in EVE ETR seeds, ethylene production

temporarily decreased after radicle protrusion, and then increased again. Guzman and Ecker (1990) reported that different tissues of ethylene insensitive *Arabidopsis* plants produced higher amounts of ethylene than WT tissues. The reason for the ethylene overproduction in unknown. It is possible that ethylene production in lettuce seeds during germination is auto-catalytic. Petuzzellii et al. (2000) reported that during pea germination ethylene promoted its synthesis through positive feedback regulation of ACC oxidase. Barry et al. (2003) reported that an increase in expression of ethylene dependent ACC synthase genes was delayed in *Nr* fruits as compared to WT tomato fruits

Ethylene production in dark, at both 20ºC and 36ºC was similar or reduced in EVE ETR lines as compared to WT EVE seeds. This indicated that light stimulated germination of EVE ETR seeds, regardless of temperature. Such stimulation by light was observed in DGB and EVE seeds matured at 30º/20ºC (chapter 4) and was hypothesized to be related to direct or indirect stimulation of ACC synthase or ACC oxidase, or both. Although ethylene production by EVE ETR 6-7 seeds was similar to that of WT EVE seeds at 36ºC, EVE ETR 6-7 germination was reduced indicating that the reduced ability to perceive ethylene reduced EVE ETR ability to germinate at the supraoptimal temperature in dark. EVE ETR 2-20 seeds appeared to produce less ethylene at 36 ºC than WT EVE seeds. However, it was possible that they could still produce the same amount of ethylene as WT EVE seeds but the amount of detectable ethylene was low due to the lower number of germinated seeds. Nascimento (1998) hypothesized that ethylene might be 'trapped' prior to germination in lettuce seeds, which would result in detection of low ethylene levels prior to germination and would underestimate ethylene production if germination was low.

Germination and ethylene production behavior of EVE ETR 18-1 seeds were different from that of the other two EVE ETR lines. EVE ETR 18-1 seeds produced a similar amount of ethylene as WT EVE seeds in light and at 20ºC in dark, and lower amount of ethylene than WT EVE seeds at 36ºC in dark (Tables 6-9 and 6-10). Regardless, their germination was 100% under all experimental conditions. A possible explanation for this may be that seeds from EVE ETR 18-1 line have higher sensitivity to ethylene than seeds from EVE ETR 2-20 and EVE ETR 6-7 lines. This might have resulted simultaneously in their higher germination and reduced ethylene production as compared to the other ETR lines.

Ethylene production and germination data indicated that there was more ethylene required for completion of germination at supraoptimal (36ºC) than at optimal (20ºC) temperature. WT EVE seeds produced an average of 31.5 nl/g /h ethylene at 36ºC in dark as compared to 13.1 nl/g/h/ at 20ºC in dark. Under both conditions there was 100% germination. The phenology of both EVE ETR 2-20 seeds and WT EVE seeds germinated at 20°C and 36°C also confirmed that more ethylene was produced at 36°C than at 20°C. The seedlings germinated at 36°C were shorter and with swollen hypocotyls (Figure 6-9). The latter has been described as effect of production of high amounts of ethylene (Abeles, 1986). These data also serve as evidence that imbibition in light results in production of more ethylene than is required for germination. At 20ºC, WT EVE seeds germinated at 100% in both light and dark. However, they produced around 3 times more ethylene in light than in dark.

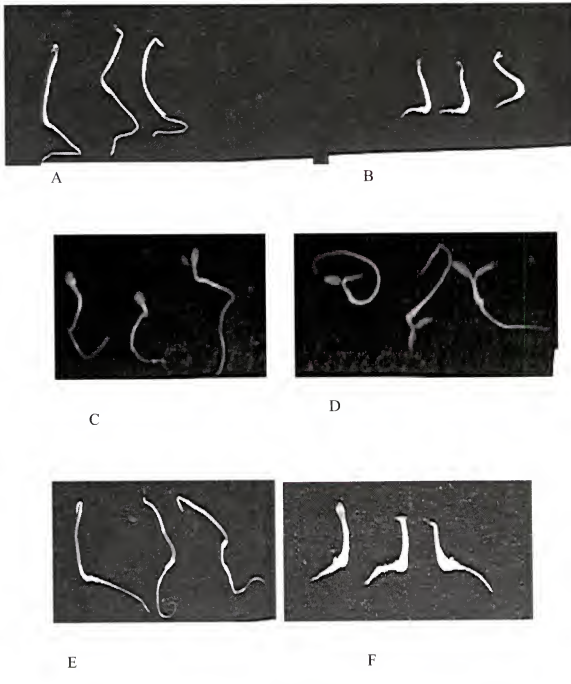


Figure 6-9 Phenology of EVE ETR 2-20 and WT EVE seedlings grown at 20°C and 36°C in dark or light (only wt EVE) for 5 days. Note that the seedlings grown at 36°C were shorter and with swollen hypocotyls as compared to seedlings grown at 20°C. The difference was observed in both light and dark but was more prominent in dark. A) EVE ETR 2-20 at 20°C in dark. B) EVE ETR 2-20 at 36°C in dark. C) WT EVE at 20°C in light. D) WT EVE at 36°C in light. E) WT EVE at 20°C in dark. F) WT EVE at 36°C in dark.

Germination of Third Generation (T3) Ethylene Insensitive EVE ETR Seeds

The different effects of reduced ethylene perception on germination, rate of germination, and ethylene production, in thermosensitive DGB and thermotolerant EVE seeds illustrated the complexity of the germination process in lettuce. In order to better correlate ethylene perception with lettuce germination, third (T3) generation EVE ETR seed lots were produced. In order to obtain these seed lots, the seeds from the individual plants from the separate lines (EVE ETR 2-5, 2-19 and 2-20 for line EVE ETR 2; EVE ETR 6-6 and 6-7 for EVE ETR 6 line; and EVE ETR 18-1 and 18-5 for EVE ETR 18 line) together with WT EVE seeds were grown. The seeds coming from the individual T2 plants were combined and seeds were pooled from the lines. Seeds coming from azygous plants (EVE ETR 2-6 and EVE ETR 2-12) were included as controls along with WT.

Germination of all ethylene insensitive and WT EVE seeds was 100% at 20°C in light (Table 6-11). At 36°C, EVE ETR 18-1 and EVE ETR 18-5 had significantly lower germination that the other ETR or WT lines. EVE ETR 18-5 had significantly higher germination than EVE ETR 18-1.

In dark, germination of all EVE ETR and WT EVE seeds was at or close to 100% at 20°C (Table 6-12). Germination of all seeds was significantly reduced at the supraoptimal temperature. The average germination percent at 36°C was 18% while that at 20°C was 98%. The ETR and WT seeds germinated the same at each temperature.

There was no difference in the speed of germination between T3 ethylene insensitive EVE seeds and WT EVE seeds at 20°C (Figure 6- 10).

Table 6-11 Germination of T3 WT and ethylene insensitive EVE seeds at 20°C and 36°C in light

Genotype	Temperature	
	20°C	36°C
EVE ETR 2-5	100.0	100.0
EVE ETR 2-19	100.0	100.0
EVE ETR 2-20	100.0	98.9
EVE ETR 6-6	100.0	100.0
EVE ETR 6-7	100.0	97.8
EVE ETR 18-1	98.9	76.7
EVE ETR 18-5	95.6	93.3
WT EVE	100.0	97.8
EVE ETR 2-6	100.0	98.9
EVE ETR 2 -2	100.0	97.8
LSD Value	5.8	

Data were analyzed using ANOVA. Means were separated using Least Significant Difference (LSD) for the two way interaction, temperature and genotype.

Table 6-12 Germination of T3 WT and ethylene insensitive EVE seeds at 20°C and 36°C in dark

Genotype	Temperature	
	20°C	36°C
EVE ETR 2-5	97.8	20.0
EVE ETR 2-19	97.8	20.0
EVE ETR 2-20	98.9	11.1
EVE ETR 6-6	97.8	12.2
EVE ETR 6-7	98.9	15.6
EVE ETR 18-1	100.0	12.2
EVE ETR 18-5	94.4	23.3
WT EVE	96.7	24.5
EVE ETR 2-6	100.0	17.8
EVE ETR 2-12	98.9	22.2
Mean (temperature)	98.1	17.9**

Data were analyzed using ANOVA. ** indicates significance at P=0.01.

Regardless of the imbibition conditions, few seeds germinated at 12 hours and germination of all seeds was almost complete at 20 hours. At 36°C, in light, the seeds from all lines except EVE ETR 18-1 and EVE ETR 18-5 started to germinate at 10 hours and reached maximal germination at 16 hours. EVE ETR 18-1 and EVE ETR 18-5 seeds germinated more slowly, reaching maximal germination at 48 hours for EVE ETR 18-1 and 36 hours for EVE ETR 18-5 (Figure 6-10).

The ethylene production of third generation EVE ETR seeds during imbibition at 20°C and 36°C in dark was measured. There were no differences between the germination of the ETR seeds and WT seeds at either temperature (Table 6-13). All seeds responded with a similar decrease in germination to the increase in temperature from 20°C to 36°C. The difference between the overall germination at 20°C and 36°C was significant, 99% at 20°C as compared to 51% at 36°C (Table 6-13). At 20°C, although all seeds had similar, close to 100% germination, EVE ETR seeds produced significantly more ethylene than WT EVE seeds. At 36°C, EVE ETR 6-7 and EVE ETR 18-1 seeds produced more ethylene than WT EVE seeds (Table 6-13).

Germination and ethylene production over the course of 48 hours of imbibition for EVE ETR 2-20 and WT EVE seeds are represented in Figure 6-11. At 20°C, EVE ETR 2-20 germination started at 12 hours and reached 100% at 24 hours. Ethylene production, however, increased steadily throughout the whole imbibition, reaching maximum at 48 hours. At 36°C, both the EVE ETR 2-20 germination and ethylene production were lower than those at 20°C (Figure 6-11).

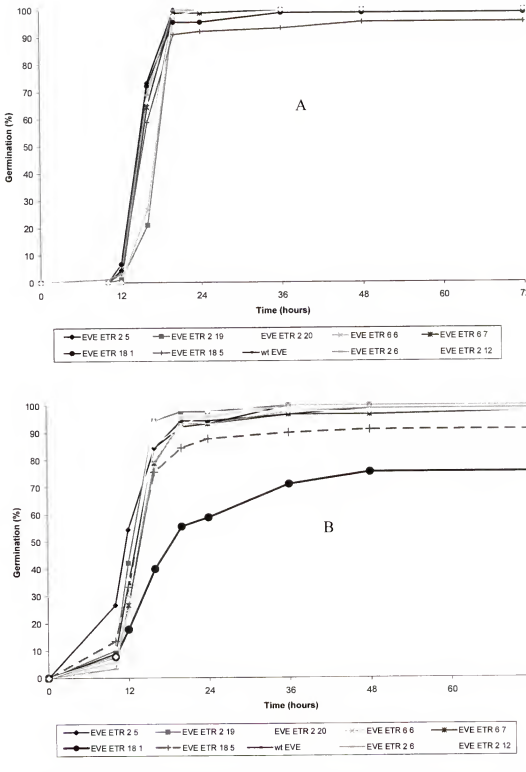
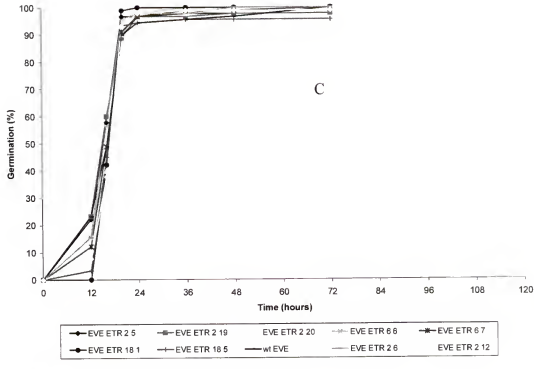


Figure 6-10 Speed of germination of T3 EVE ETR and WT EVE seeds. A) 20ºC in light. B) 36ºC in light. C) 20ºC in dark. D) 36ºC in dark.



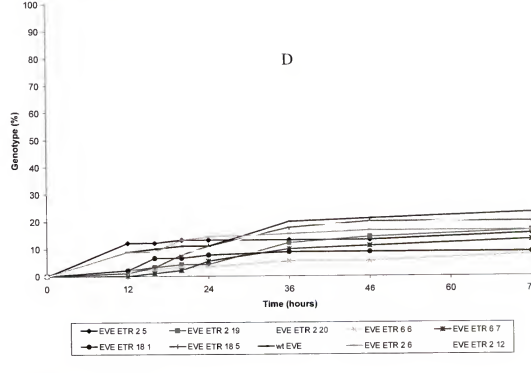


Figure 6-10. Continued.

Table 6-13 Germination and ethylene production of T3 EVE ETR and WT EVE seeds in dark, at 20ºC and 36ºC

Genotype	Germination (%)		Ethylene (nl/g/h)	
	Temperature		Temperature	
	20°C	36°C	20°C	36°C
EVE ETR 2-20	98.9	24.4	17.72	9.50
EVE ETR 6-7	97.8	62.3	17.04	17.00
EVE ETR 18-1	100.0	61.1	16.86	12.75
WT EVE	98.9	54.5	11.11	8.06
Means (temperature)	98.9	50.6**		
LSD Value			3.8	

Data were analyzed using ANOVA. The means were separate using Least Significant Difference (LSD) values. The values for ethylene production were calculated by averaging ethylene production over 48 hours of seed imbibition. Ethylene was evaluated every three hours.

At 20°C, WT EVE germination also started at 12 hours but was completed earlier than EVE ETR 2-20 germination, at 20 hours. At this temperature, WT EVE ethylene production had distinct peak at 24 hours. At 36°C, both germination and ethylene production of WT EVE seeds increased during the whole imbibition and obtained highest levels at 48 hours (Figure 6-11).

Although germination of third generation WT EVE seeds was as low as EVE ETR germination at 36°C in dark, EVE ETR seeds produced significantly more ethylene than WT EVE seeds. This indicated that more ethylene was required for germination in the ethylene insensitive seeds than in WT seeds which confirmed the effect of the partial ethylene insensitivity on EVE germination at supraoptimal temperature in dark. A similar situation was observed with germination of the second generation EVE ETR seeds in light where there was no difference in germination between all the lines but EVE ETR seeds produced significantly more ethylene than WT EVE seeds.

Both germination and ethylene production of WT EVE and EVE ETR seeds at 36°C in dark were much lower in the third generation than in the second generation. As seen from Table 6-1, EVE seeds from the two generations were produced at different environmental temperatures. It is very possible that the different production conditions accounted for their different ability to produce ethylene, and, consequently, to germinate.

As already discussed in chapter 4, the temperatures during seed maturation can affect ethylene production and, consequently, germination of both DGB and EVE seeds. Depending on the temperatures during maturation, thermosensitive seeds can behave like thermotolerant and thermotolerant as thermosensitive.

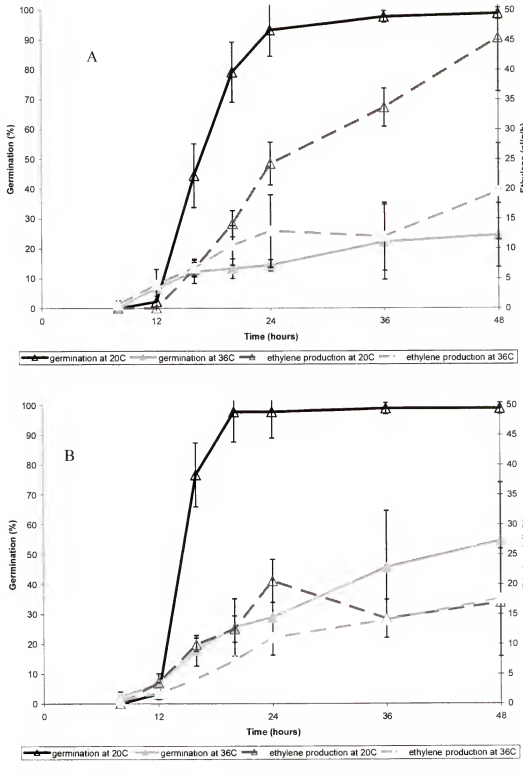


Figure 6-11 Germination (solid lines) and ethylene production (broken lines) of T3 EVE ETR 2-20 and WT EVE seeds imbibed at 20ºC (open triangles) and 36ºC (filled triangles) in dark. Vertical bars represent standard errors. A) EVE ETR 2-20. B) WT EVE.

Germination and ethylene determination experiments conducted with T2 DGB ETR seeds and T2 and T3 EVE ETR seeds demonstrated that reduced ethylene perception affected lettuce germination. However, the effect of reduced ethylene perception on germination was negated at some cases (T2 EVE ETR germination at 36°C in light and T3 germination at 36°C in dark) by higher production of ethylene by the ETR lines than WT. It would be beneficial to produce seeds from the different EVE and DGB ETR lines under different temperature conditions and subject them to different durations of after-ripening prior to germination in order to examine the interaction between reduced ethylene perception and environmental factors in determining their germination potential. Such experiments would also further explore the differences between the different transgenic lines. It was noticed that, for example, EVE ETR 6-7 seeds consistently produced more ethylene and had higher germination than EVE ETR 2-20 seeds.

Germination of Transgenic Seeds with Altered Ethylene Production

Since all the results presented previously (chapters 3 and 4) and also by other authors (Prusinski and Khan, 1990; Nascimento et al., 2000) suggest that ethylene may regulate lettuce germination in a quantitative manner, meaning that when more ethylene is produced, germination is higher, it was decided to obtain transgenic EVE and DGB seeds that overexpress ACC synthase. However, such high ethylene production might have had a negative effect on plant development that resulted in suppression, rather than promotion of ethylene synthesis (see chapter 5). One DGB and two EVE lines with reduced ethylene production (DGB ACC 2, EVE ACC 1, and EVE ACC 4) and one EVE line with higher than the WT ethylene production (EVE ACC 3; see chapter 5) were used

in germination experiments. Since the number of seeds from the EVE ACC ethylene overproducing line (EVE ACC 3) was low, their germination was only tested at supraoptimal temperature (36°C).

There was no difference between DGB ACC 2-4, DGB ACC 2-7 and WT DGB germination in light or dark (Table 6-14). There was also no difference between the germination in light or dark for any of the seed lots. All seeds germinated significantly better in water than in 20 mM STS, regardless of the imbibition conditions.

When the seeds were imbibed at supraoptimal temperature (36°C), there were significant differences in the percent germinated seeds among the different transgenic lines (Table 6-15). In light, WT DGB seeds had greater germination than either DGB ACC 2-4 or DGB ACC 2-7 seeds. In dark, germination of all DGB seeds was completely inhibited. Application of 20 mM STS completely inhibited DGB germination (Table 6-15).

In light, EVE ACC 3 and WT EVE germinations were similar (above 84%). In dark, EVE ACC 3 seeds had significantly greater germination that WT EVE seeds, 44% as compared to 11%. Application of 20 mM STS inhibited both EVE ACC 3 and WT EVE germination in either light or dark. This indicated that regardless of the suspected production of more ethylene by EVE ACC 3 seeds than by WT EVE seeds, the effect of imposed reduction in ethylene perception was similar (Table 6-15).

There was no difference in the speed of germination among the seeds from the two DGB ACC 2 plants (DGB ACC 2-4 and DGB ACC 2-7) and WT DGB seeds at 20°C in light (Figure 6-12).

Table 6-14 Germination of WT DGB and T2 DGB ACC seeds in water or 20 mM STS, in dark or light, at 20°C

Genotype	Water		STS	
	Light	Dark	Light	Dark
DGB ACC 2-4	100.0	100.0	0.0	0.0
DGB ACC 2-7	100.0	91.1	0.0	0.0
WT DGB	100.0	95.6	0.0	0.0
Means (Solutions)		97.8		0.0**

Data were analyzed using ANOVA. The means for the two solutions were separated using LSD. ** represents significance at P=0.01.

Table 6-15 Germination of WT DGB and T2 DGB ACC seeds in water or 20 mM STS, in dark or light, at 36°C

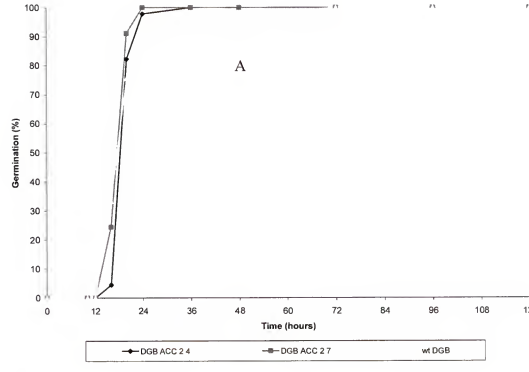
Genotype	Water		STS	
	Light	Dark	Light	Dark
DGB ACC 2-4	4.4	0.0	2.2	0.0
DGB ACC 2-7	0.0	2.2	0.0	0.0
WT DGB	22.2	0.0	0.0	0.0
EVE ACC 3	93.3	44.4	4.4	0.0
WT EVE	84.4	11.1	0.0	0.0
LSD Value		13.2		

Data were analyzed using ANOVA. The means for the three way interaction genotype X solutions X light conditions were separated using LSD.

All seeds started to germinate at 16 hours after the initiation of imbibition and reached 100% germination by 24 hours. At 36ºC, in light, WT EVE seeds started to germinate earlier than EVE ACC 3 seeds, at 10 hours as compared to 12 hours. WT EVE seeds reached maximal germination earlier, at 20 hours as compared to 36 hours for EVE ACC 3 (Figure 6-12). Although WT EVE germination was faster that EVE ACC 3 germination, EVE ACC 3 had higher final germination percent than WT EVE.

In dark, at 20ºC, WT DGB germination was more rapid than DGB ACC 2-4 and 2-7 germination. WT DGB seeds started to geminate at 16 hours and completed germination by 20 hours. In comparison, DGB ACC 2-7 seeds started to germinate at 20 hours and completed germination by 24 hours. At 36ºC, in dark, WT EVE germination started at 20 hours and increased throughout the imbibition to reach a maximum (11%) at 120 hours (Figure 6-12). EVE ACC 3 germination began at 12 hours and reached maximum (44%) by 24 hours.

Ethylene production and germination during the first 48 hours of imbibition was measured for 5 ACC lines (DGB ACC 2-4, DGB ACC 2-7, EVE ACC 1-8, EVE ACC 4-13, and EVE ACC 3) and two controls (WT DGB and EVE) (Tables 6-16, 6-17). At 20ºC, DGB ACC 2-4 and 2-7 seeds and WT DGB seeds germinated 100% in both light or dark (Table 6-16). When the temperature was increased to 36ºC, DGB ACC and WT DGB germination were completely inhibited. Ethylene production of DGB ACC 2-4, DGB ACC 2-7, and WT DGB seeds was similar at 20ºC, in both light and dark. However, ethylene production by all DGB seed lots was reduced in dark as compared to light (Table 6-17). At 36ºC all DGB seed lots produced very little ethylene.



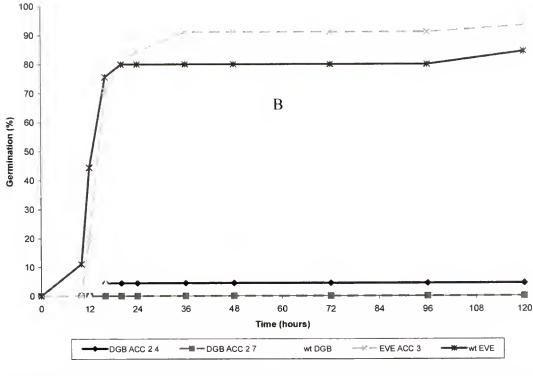
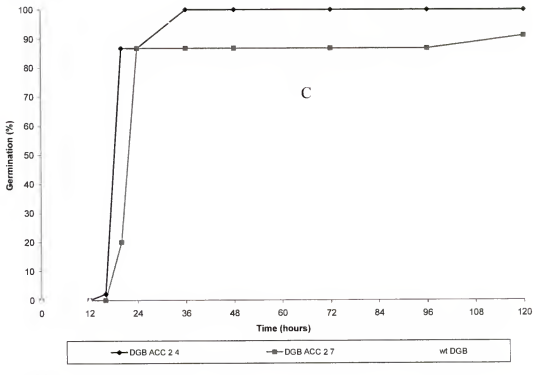


Figure 6-12 Speed of germination of T2 DGB and EVE ACC seeds. A) 20°C in light. B) 36°C in light. C) 20°C in dark. D) 36°C in dark.



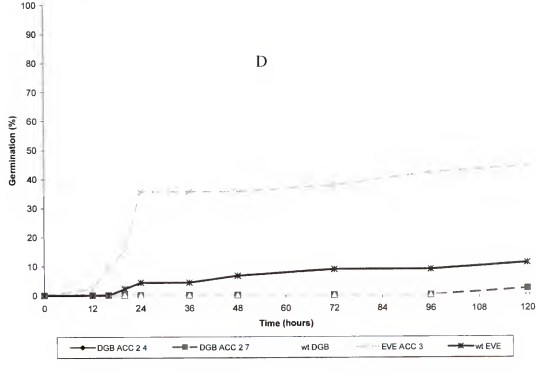


Figure 6-12. Continued.

Table 6-16 Germination of WT DGB and DGB ACC seeds at 20ºC and 36ºC, in light and dark

Genotype	Light Conditions/Temperature			
	Light		Dark	
	20ºC	36ºC	20ºC	36ºC
DGB ACC 2-4	100.0	1.1	100.0	1.1
DGB ACC 2-7	100.0	3.3	100.0	0.0
WT DGB	100.0	6.7	100.0	1.1
LSD Value		6.5		

Table 6-17 Ethylene production (nl/g/h) of WT DGB and DGB ACC seeds at 20°C and 36°C, in light and dark

Genotype	Light Conditions/Temperature			
	Light		Dark	
	20°C	36°C	20°C	36°C
DGB ACC 2-4	23.99	0.86	8.36	1.06
DGB ACC 2-7	28.73	0.77	7.59	0.36
WT DGB	26.28	4.20	8.15	0.45
LSD Value		4.6		

Data were analyzed using ANOVA. The means were separate using Least Significant Difference (LSD) values. The values for ethylene production were calculated by averaging ethylene production over 48 hours of seed imbibition. Ethylene was evaluated every three hours.

EVE ACC seeds (EVE ACC 1-8, EVE ACC 4-13, and EVE ACC 3) and WT EVE seeds also germinated at 100% in either light or dark at 20ºC (Table 6-18). At 36ºC, in light, all EVE seed lots again germinated close to 100%. However, imbibition at 36ºC in dark, significantly reduced germination of all EVE seeds as compared to that in light (Table 6-18). EVE ACC 3 germination was greater than germination of all other EVE ACC lines and WT EVE. At 36ºC, in dark, WT EVE seeds had higher germination than EVE ACC 1-8 seeds (Table 6-18). During germination at 20°C, in both light and dark, all EVE ACC lines and WT EVE produced similar amounts of ethylene (Table 6-19). At 36°C, in light, all EVE seeds produced more ethylene than at 20°C. EVE ACC 3 seeds produced more ethylene than any other EVE seed lots (Table 6-19). At 36°C, in dark, ethylene production by all EVE seeds was lower than their ethylene production at 36°C in light. However, EVE ACC 3 seeds still produced more ethylene than all other EVE seeds (Table 6-19).

The ethylene determination experiments conducted with T1 ACC seeds provided additional support for the importance of ethylene in lettuce germination. The amounts of ethylene produced by DGB ACC 2 seeds and WT DGB seeds were similar under all experimental conditions as was their germination (Tables 6-16, 6-17). EVE ACC 3 seeds produced more ethylene than WT EVE seeds during germination at 36°C, while ethylene production of EVE ACC 1-8 and EVE ACC 4-13 seeds at this temperature was lower than that of WT EVE (Table 6-19). This observation strengthened the hypothesis that EVE ACC 3 was a line producing more ethylene than WT EVE and that ethylene production in EVE ACC 1 and EVE ACC 4 lines might be suppressed.

Table 6-18 Germination of WT EVE and EVE ACC seeds at 20ºC and 36ºC, in light and dark

Genotype	Light Conditions/Temperature			
	Light		Dark	
	20ºC	36ºC	20ºC	36ºC
EVE ACC 1-8	100.0	100.0	100.0	7.8
EVE ACC 4-13	98.9	100.0	100.0	10.0
EVE ACC 3	100.0	95.6	100.0	52.2
WT EVE	100.0	96.7	100.0	15.6
LSD Value		6.5		

Table 6-19 Ethylene production (nl/g/h) of WT EVE and EVE ACC seeds at 20°C and 36°C, in light and dark

Genotype	Light Conditions/Temperature			
	Light		Dark	
	20°C	36°C	20°C	36°C
EVE ACC 1-8	27.63	53.88	8.31	2.46
EVE ACC 4-1	32.09	61.50	11.20	2.29
EVE ACC 3	27.29	79.77	9.10	9.86
WT EVE	36.62	60.65	10.57	4.21
LSD Value		4.6		

Data were analyzed using ANOVA. The means were separate using Least Significant Difference (LSD) values. The values for ethylene production were calculated by averaging ethylene production over 48 hours of seed imbibition. Ethylene was evaluated every three hours.

All EVE seeds produced more ethylene at 36°C in light than at 20°C. This observation supports the hypothesis that germination at supraoptimal temperature requires higher amounts of ethylene than germination at optimal temperature. The reason for this suspected increase in requirement is unknown. It may be related to the necessity of overcoming a block of germination at the supraoptimal temperature. The data obtained from experiments with ACC seeds also confirmed that imbibition in dark led to reduction in ethylene production in both DGB and EVE seeds, at both temperatures.

It may be possible to obtain more transgenic lines with a stronger increase in ethylene production, if a seed-specific promoter, or even better, germination-specific promoter, is used. This will allow the effect of the increased ethylene production to be observed in more transgenic lines and will provide more information about the amount of ethylene important for germination under different experimental conditions.

Summary

Reduction in the sensitivity to ethylene in thermosensitive 'Dark Green Boston' (DGB) seeds and thermotolerant 'Everglades' (EVE) seeds reduced the ability of these seeds to germinate at optimal or supraoptimal temperatures. Germination of DGB seeds was reduced at lower temperatures than WT DGB germination in light. Both second generation (T2) EVE ETR seeds and WT EVE seeds had 100% germination at 20°C and 36°C in light and 20°C in dark. At 36°C in dark, T2 EVE ETR germination was significantly reduced as compared to WT EVE germination. The reason for the lack of effect of reduced ethylene perception on EVE germination at 36°C in light was the production of more ethylene by the EVE ETR seeds than WT EVE seeds. This high

ethylene production might have compensated for the reduction in ethylene perception. Since ethylene production was significantly reduced in dark as compared to light, the amount of ethylene produced might have been not sufficient to overcome the reduced ethylene perception of T2 EVE ETR seeds during germination at 36°C in dark. The reason for the promotive effect of light on ethylene production is unknown. Light may stimulate some of the steps in ethylene biosynthesis. Third generation (T3) EVE ETR seeds had similar germination to WT EVE seeds under all experimental conditions, optimal or supraoptimal temperatures, light or dark. The reason for this was the production of significantly more ethylene in T3 EVE ETR seeds than in WT EVE seeds. The experiments conducted with T2 and T3 EVE ETR seeds demonstrate that the ability of lettuce seeds to germinate, especially under stressful conditions, is determined by the balance between ethylene perception and ethylene production and increase in ethylene production can compensate for decreased perception. Studies with DGB and EVE ACC plants demonstrated that if lettuce seeds had normal (WT) ethylene perception, their germination at supraoptimal temperature, was solely determined by the amount of ethylene produced.

APPENDIX

Table A-1 Correlation of germination (%) and ethylene production (nl/g/h) in DGB and EVE seeds matured at 30°/20°C and 20°/10°C during imbibition at 20°C or 36°C, in light or dark

Imbibition	conditions	DGB20/10	DGB3020	EVE2010	EVE3020
Light	20°C	0.85**	0.70**	0.93**	0.85**
	36°C	0.97**	0.79**	0.93**	0.60**
Dark	20°C	0.96**	0.73**	0.88**	0.87**
	36°C	0.89**	0.88**	0.91**	0.89**

Correlation was done using Pearson correlation coefficient Means for calculating the correlation coefficients for the dependent and independent variable were derived by averaging the germination and ethylene production for all the duration of imbibition.

Table A-2 Correlation of germination (in %) and ethylene production (nl/g/h) of WT and DGB ETR seeds during germination at 20°C and 36°C, in light or dark

Imbibition conditions		DGB ETR 6	DGB ETR 10	DGB ETR 14	WT DGB
Light	20°C	0.93**	0.85**	0.86**	0.78**
	36°C	0.57*	0.69**	0.51NS	0.70**
Dark	20°C	0.97**	NA	0.96**	0.86**
	28°C	0.87**	NA	0.75**	0.87**

Correlation was done using Pearson correlation coefficient P with N=20 for light imbibition and N=12 for dark imbibition. ** indicates significance of the correlation at P=0.01 and * indicates significance at P=0.05. Means for calculating the correlation coefficients were derived by averaging the germination and ethylene production for the whole imbibition period.

Table A-3 Correlation of germination (%) and ethylene production (nl/g/h) of WT and EVE ETR seeds during germination at 20°C and 36°C, in light or dark

Imbibition conditions		EVE ETR 2-20	EVE ETR 6-7	EVE ETR 18-1	WT EVE
Light	20°C	0.82**	0.82**	0.65**	0.74**
	36°C	0.72*	0.86**	0.75**	0.75**
Dark	20°C	0.91**	0.81**	0.71**	0.71**
	36°C	0.79**	0.71**	0.58**	0.56**

Correlation was done using Pearson correlation coefficient P with N=21 for light imbibition and N=18 for dark imbibition. ** indicates significance at P=0.01 and * indicates significance at P=0.05. Means for calculating the correlation coefficients were derived by averaging the germination and ethylene production for the whole imbibition period.

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BIOGRAPHICAL SKETCH

Iwanka Kozarewa was born on April 8, 1976, in Sofia, Bulgaria. She got her Master of Science degree in July 1999 in molecular biology with specialty in plant genetics from the University of Sofia. Afterwards, I. Kozarewa started a PhD program at the University of Florida, Horticultural Sciences Department. Her research was focused on lettuce seed thermodormancy, especially the role of ethylene in circumventing it. Her future plants are to continue working in the area of seed and vegetative dormancy, with special emphasis on interactions between light, plant hormones and environmental growth conditions.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Daniel J. Cantliffe, Chair
Professor of Horticultural Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Russell T. Nagata
Associate Professor of Horticultural Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Donald J. Huber
Professor of Horticultural Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Harry J. Klee
Eminent Scholar of Horticultural Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Maria Gallo-Meagher
Associate Professor of Agronomy

This dissertation was submitted to the Graduate Faculty of the College of Agricultural and Life Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 2004

Dean, College of Agricultural and Life Sciences

Dean, Graduate School